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A Comparative Ultrastructural Study of In Vivo and In Vitro Developed Late Preimplantation Mouse Blastocysts

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A COMPARATIVE ULTRASTRUCTURAL STUDY OF IN VIVO AND IN VITRO
DEVELOPED LATE PREIMPLANTATION MOUSE BLASTOCYSTS

by

Hal D. McReynolds

A Dissertation Submitted to the Faculty of the
Graduate School of Loyola University
in Partial Fulfillment of the
Requirements for the
Degree of
Doctor of Philosophy

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BIOGRAPHY

Hal D. McReynolds was born in Bloomington, Illinois on January 5, 1941. In 1959, he graduated from McLean-Waynesville High School in McLean, Illinois, and then continued his studies at the University of Notre Dame, Notre Dame, Indiana, and the University of Illinois at Urbana. He received the Bachelor of Science degree in Zoology in June, 1965, from the University of Illinois.

In the Fall of 1965, the author began his graduate studies in the Department of Anatomy, Loyola University Stritch School of Medicine, and in the Spring of 1966, he chose Dr. Robert Hadek as his faculty advisor. Since 1966, Mr. McReynolds has been a Graduate Teaching Assistant in the Department of Anatomy.

In June, 1967, Hal D. McReynolds received the Master of Science degree from Loyola University of Chicago, and in May, 1969, he was elected an Associate Member of the Society of Sigma Xi. He is also a member of the American Association for the Advancement of Science.

Hal D. McReynolds was married to Kathie Ann Yeast on June 18, 1966, and they have a daughter, Amy Kathleen, who was born on August 27, 1968.

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ABSTRACT

The fine structure of superovulated late mouse blastocysts developed for 72 or 92 hours in vitro from the late two-cell stage, as well as that of superovulated blastocysts developed 26 hours in vitro from the late morula or early blastocyst stage has been compared with the ultrastructure of the superovulated in vivo developed late preimplantation mouse blastocyst collected 100 to 108 hours after ovulation,

Cell nuclei of the in vivo developed late preimplantation blastocyst contain well-differentiated nucleoli, moderate amounts of heterochromatin, and ribosome-lined outer nuclear membranes often are observed in direct continuity with cisternae of rough endoplasmic reticulum. Cells of the in vivo developed late blastocyst contain numerous ribosomes, moderate amounts of rough endoplasmic reticulum, and high resolution autoradiography suggests that the blastocysts are actively incorporating amino acids. The Golgi complex appears rather quiescent. Mitochondria are pleomorphic and display an electron-dense matrix, and they appear to contain numerous cristae. Lysosome-like bodies are present in both cell types but crystalloids and fibrous strands are rarely observed.

The inner cell mass of the in vivo developed late preimplantation mouse blastocyst has the ability to differentiate a layer of endoderm cells. This represents one of the first ultrastructural indications of differentiation among the cells of the preimplantation mouse embryo.

Mouse blastocysts developed 72 hours in vitro from the late two-cell

stage appear less differentiated in several aspects. Nucleolar fine structure varies from spherical undifferentiated to moderately differentiated, and little condensed chromatin is found within the nucleus. In addition, nuclei contain annulate lamellae which are characteristic of earlier cleavage stages. Cells of these blastocysts appear to contain fewer ribosomes and elements of rough endoplasmic reticulum, and the Golgi complex is somewhat distended. Swollen mitochondria with multiple intracristal vacuoles are often observed in intimate association with channels of rough endoplasmic reticulum. Crystalloids and fibrous inclusions are present in large numbers, and lysosome-like and myelin-like figures are also observed.

Although cells of blastocysts cultured 92 hours in vitro from the late two-cell stage appeared to contain more ribosomes and elements of rough endoplasmic reticulum than those of embryos cultured 72 hours in vitro, many of their mitochondria also appear swollen. The Golgi complex is usually moderately distended and lysosome-like structures are commonly observed. Crystalloids remain numerous but fibrous strands are somewhat reduced in number. Nuclei of blastocysts cultured 92 hours in vitro appear somewhat more differentiated than those of embryos cultured 72 hours in vitro from the two-cell stage. A large number of cells of embryos developed 92 hours in vitro appeared to be in different stages of autolysis. There was no evidence of endoderm cell differentiation in blastocysts developed 72 or 92 hours in vitro.

Cell nuclei of blastocysts developed 26 hours in vitro from the late morula or early blastocyst stage appear well-differentiated and they closely resemble those of the in vivo developed late preimplantation mouse blastocyst.

Numerous ribosomes and moderate amounts of rough endoplasmic reticulum are present in the cytoplasm of trophoblast and inner mass cells, and mitochondria are similar to those of the in vivo developed late preimplantation blastocyst. The fine structure of these blastocysts differs from that of the in vivo developed late blastocyst in that the Golgi complex is somewhat distended, crystalloids are numerous, and fibrous strands are occasionally observed. As in the in vivo developed late blastocyst, the inner cell mass is able to differentiate a layer of endoderm cells.

Cell surface modifications such as microvilli, junctional complexes, and micropinocytotic caveolae were observed in all blastocysts examined.

Possible reasons for the apparently less differentiated state of blastocysts developed 72 hours in vitro from the late two-cell stage include (1) the effect of the sudden transition from in vivo to in vitro developmental conditions, (2) it may be more difficult for the embryos to accomodate important changes in metabolic pathways during in vitro preimplantation development, and (3) the absence of certain important maternal "factors", as well as hormonal stimulation when development takes place outside the maternal reproductive tract.

I. INTRODUCTION

A. ORIGIN OF THE FEMALE GAMETE

The primordial germ cells of vertebrates arise extragonadally and migrate to the site of the future gonads (16,42,56). In the mouse, all definitive germ cells are derived from fewer than 100 cells which are first observed in the yolk sac endoderm near the allantoic diverticulum during the eighth day of gestation (166). The germ cells actively migrate along the dorsal mesentery and enter the paired genital ridges. By the twelfth day of gestation the germ cell number has multiplied to nearly 5,000 (128).

In the mouse, mitotic activity of oogonia is completed approximately five days before birth (114) and most germ cells are in prophase of the first meiotic division and are called primary oocytes (137). Numerous primary oocytes are in the pachytene stage of the first meiotic prophase at fetal days 16 and 17 (124), and they may enter the dictyate or nuclear resting stage by birth. Nuclei of primary oocytes remain in the dictyate stage until just prior to ovulation.

By the eighteenth fetal day or the first postnatal day of development in the mouse, most primary oocytes are surrounded by flattened follicular cells to form primordial unilaminar follicles (137,138). By the second day after birth the primary oocytes are surrounded by a single layer of cuboidal or low columnar cells, and they are called unilaminar primary follicles (137).

Some bilaminar primary follicles may also be present at this time.

Primary oocytes remain in the dictyate stage of the first meiotic division until puberty when those oocytes which are to be ovulated are activated by pituitary gonadotropins (53). Within five to eight hours after stimulation by luteinizing hormone (LH), the dictyate chromatin condenses, the primary oocyte completes the first meiotic division to become a secondary oocyte, and the first polar body is extruded. The secondary oocyte is ovulated six to nine hours later (50). Nuclear progression from the dictyate phase to metaphase II, known as the first maturation division, has been detailed in mouse oocytes in vitro (47).

The ovulated secondary oocyte is surrounded by an acellular layer of glycoprotein, the zona pellucida, which was formed in the ovarian follicle. The zona pellucida itself is surrounded by a layer of follicular or granulosa cells which also originated in the ovarian follicle. In the rabbit, it has been shown that the innermost layer of these cells (corona radiata) may be important in assisting sperm penetration of the secondary oocyte (40).

Mature secondary oocytes of the mouse have a diameter of approximately 95 microns when the outer diameter of the zona pellucida is considered, but the diameter of the vitellus is approximately eighty microns (109).

B. FERTILIZATION AND CLEAVAGE

If mating has occurred, spermatozoa are present in the ampullary region of the oviduct at the time of ovulation (109). The secondary oocyte is assumedly fertilized by the first spermatozoon to reach it (166), and as a

rule, it is penetrated prior to dissolution of the corona radiata (50,109). It is assumed that hyaluronidase, contained in the acrosomal cap of the spermatozoon, may enable the male gamete to penetrate the matrix between the corona cells (19,107).

Although a number of theories are extant with regard to the prevention of polyspermy (5,18,166), under physiological conditions only one spermatozoon is incorporated into the cytoplasm of the egg (4,6,83). After the spermatozoon is incorporated into the secondary oocyte, the second polar body is extruded into the perivitelline space, the male and female pronuclei form, and the secondary oocyte becomes a mature ovum (166).

Normal preimplantation development in the mouse is completed within five days after mating, and it can be divided into the following stages: fertilized ovum, two-cell, four-cell, eight-cell, sixteen-cell, morula, early and late blastocyst (109). The first two cleavages take place while ova are still within the oviduct. The first division, occurring approximately twenty-four hours after mating, produces two cells which are nearly equal in size (109). Subsequent divisions occur at approximately twelve hour intervals (109).

Embryos with sixteen or more cells but which contain no cavity are called morulae, and they pass from the Fallopian tube into the uterus approximately sixty to seventy-two hours after fertilization (109). At the thirty-two cell stage, an eccentrically located fluid-filled cavity appears among the blastomeres. This cavity, the blastocoele, is bound by a single layer

of trophoblast cells except at one pole where cells are grouped to form an inner cell mass (109). These embryos are known as blastocysts and they settle into uterine crypts approximately four and one-half days after copulation (166).

C. THE PROBLEM

Early investigations of mammalian preimplantation development were limited by the small number of embryos which could be collected at one time. However, with the advent of hormonally-induced superovulation (50,63,69), an increasing number of experiments have been performed on preimplantation mouse embryos (9-11,13,20-33,35,50,51,64,70,74,79,90,111,123,158,163,171,178,180,181,184-186,191-195,200-202,204). Not only does superovulation allow the investigator to synchronize the estrous cycle and to accurately predict the time of ovulation in a large number of animals, it also increases the number of viable embryos which can be collected from each female (50,63,69).

For experimental purposes two sources of the "late" preimplantation mouse blastocyst have been utilized: (1) blastocysts developed in vivo for 100 to 108 hours after ovulation (24-26,28-30,33,54,64,74,92,123,125,126,129,181,184,191-195), and (2) blastocysts developed from ova which were grown in vitro from the late two-cell stage (10,13,20-23,31,32,44,51,70,78,79,119,132,163,178,180,181,184).

Although mouse blastocysts have been successfully grown in vitro in a number of relatively complex culture media (44,126,127,133), these media contain large amounts of serum or other undefined substances which make it

difficult to quantitate metabolic processes or growth requirements of the developing embryos. Therefore, the real advancement in the in vitro study of preimplantation development was Brinster's formulation of a method by which two-cell mouse ova can be developed to the blastocyst stage in vitro in a simple, chemically defined culture medium (20). Brinster claimed that development proceeded at the same rate in vitro as in vivo and that 60 to 100 per cent of the two-cell mouse ova develop to the "late" blastocyst stage following 72 hours cultivation in vitro (20). This method enables investigators to study the effects of addition or omission of substances from the culture medium during various stages of preimplantation development. For example, Brinster tested a number of possible energy sources for their ability to support development of two-cell mouse ova to the blastocyst stage in vitro (22). He determined that pyruvate, lactate, oxaloacetate, or phosphoenolpyruvate could support development when they were employed as sole energy sources in the culture medium (22). Furthermore, at its optimum concentration pyruvate was found to support development of the largest number of two-cell ova to the blastocyst stage in vitro (22).

Brinster's technique involves removal of two-cell mouse ova from the oviducts of superovulated, sexually mature female mice approximately 31 to 34 hours after the estimated time of ovulation (20,21). The embryos are then cultured 72 hours in vitro in the simple, chemically defined culture medium at 37° C in 5 per cent CO₂ in air. Hence, the post-ovulation age of these in vitro grown blastocysts is approximately the same as that of "late" preimplantation mouse blastocysts developed in vivo for 100 to 108 hours

after ovulation. Brinster's culture medium has also been used as a vehicle for experiments utilizing superovulated mouse ova grown in vivo to later stages of preimplantation development (four-cell to late blastocyst stage) and then studied in vitro for differing periods of time in the presence of radioactively labeled substances, metabolic inhibitors, enzyme substrates, energy sources, etc. (24-30,51,74,123,129,180,184,185,186,204).

Thus far, the basic parameters by which a particular culture medium is tested include: (1) whether embryonic cells remain viable for a specified period of time, (2) the occurrence of cleavage, and (3) formation of a normal-appearing blastocyst as determined by light microscopy (31). Although the latter morphological criterion is most commonly employed, the increased magnification and better resolution obtainable with the electron microscope should allow a more precise morphological basis for determining the effectiveness of this particular in vitro culture system.

In general, preimplantation development in mammals (from zygote to the late blastocyst stage) is characterized by striking changes in nuclear and cytoplasmic fine structure, including the establishment of "organelles normally associated with the rapid protein synthesis of embryonic cells" (54). These changes, which represent fine structural differentiation of the cells of the developing embryos, have been studied in detail in superovulated mouse embryos developed in vitro to the morula stage (90), as well as in superovulated mouse embryos developed in vivo to the early blastocyst stage (36). However, excepting a cursory study in which in vivo grown preimplantation blastocysts of several species (including the mouse) were compared (54),

no detailed study on the ultrastructure of the superovulated in vivo grown "late" preimplantation mouse blastocyst nor the superovulated in vitro grown "late" preimplantation mouse blastocyst has been reported. Hence, the need for a comparative ultrastructural study of superovulated in vivo and in vitro grown mouse embryos, at their latest stage of preimplantation development, was clearly indicated.

Therefore, the purpose of this investigation was: (1) to examine in detail the fine structure of the superovulated "late" preimplantation mouse blastocyst developed in vivo for 100 to 108 hours after ovulation, and (2) to compare the ultrastructure of the above with that of superovulated "late" mouse blastocysts developed in vitro for 72 hours from the late two-cell stage in Brinster's simple, chemically defined culture medium containing pyruvate as the only energy source. Such a study should indicate whether embryos grown to the blastocyst stage in vitro in such a simple culture medium are as differentiated ultrastructurally as those blastocysts which develop entirely within the maternal environment.

II. REVIEW OF RELATED LITERATURE

A. EVOLUTION OF A SIMPLE, CHEMICALLY DEFINED CULTURE MEDIUM

Early attempts to culture mouse embryos in vitro failed to produce cleavage (109,115). The first culture medium to support the development of mouse ova through several cleavage stages was that of Hammond (87), and it contained magnesium, potassium, and sodium chloride, as well as glucose and five per cent egg white. In this medium, some late four-cell and most eight-cell embryos were able to develop into blastocysts, but no two-cell ova cleaved beyond the four-cell stage.

The pH of this particular medium rose quickly to 7.8 when left exposed to the atmosphere (due to loss of CO₂ from the egg albumin) (197). Since mouse ova do not cleave in milieu whose pH is beyond 7.7 (197), Whitten formulated a buffered culture medium which contained Krebs-Ringer bicarbonate saline, glucose, egg white or albumin, and antibiotics (penicillin and streptomycin). When equilibrated with 5 per cent CO₂ in air, the pH of this medium remained constant at 7.4. Eight-cell ova cultured in Whitten's medium developed into blastocysts within 48 hours (197).

Subsequently, Whitten demonstrated that mannose, lactate, malate, or pyruvate could supply energy for in vitro development of eight-cell mouse ova to the blastocyst stage (198). In addition, some two-cell mouse ova developed

into blastocysts when a portion of the calcium chloride in the Krebs-Ringer bicarbonate saline was replaced by calcium lactate or by a pyruvate-containing salt. Later, McLaren and Biggers (120) showed that blastocysts cultured in this manner were capable of producing normal offspring when transferred to uteri of foster mothers.

Brinster (20) modified Whitten's technique by reducing the proportions of sodium chloride and calcium chloride in the culture medium while adding serum albumin and antibiotics, as well as sodium lactate for an energy source. He reported that when two-cell mouse ova are cultured in this simple medium, development proceeds at the same rate as that in vivo, and between 60 and 100 per cent of the ova develop into normal-appearing late mouse blastocysts (20). The availability of this simple medium, which permits the development of mouse ova in vitro, has greatly facilitated the study of preimplantation development in the mouse.

B. SUPEROVULATION AND PREIMPLANTATION DEVELOPMENT IN THE MOUSE

Fowler and Edwards (63) were the first investigators to show that mature mice, selected at random with regard to their estrous cycles, could be brought into estrus and induced to ovulate by treatment with gonadotropins, and that these females could carry their embryos to term. Later, Edwards and Gates (50) reported that the time of ovulation could be accurately predicted (within a three hour period) by using this method. These findings were important for studies in which the time of estrus, ovulation, and mating need to be controlled. Although the vaginal smear technique can be used to predict the time of estrus, it is less accurate in predicting the time of

ovulation, and it requires maintenance of large numbers of animals in order to select females in estrous (165). For these reasons, as well as the fact that an increased number of viable embryos can be collected at one time by using this method (50,63,69), Brinster employed superovulation in his experiments (20,21).

Realizing that an abundant supply of fertilized ova is necessary for many in vitro studies, Gates later performed experiments to determine optimum conditions for recovery of viable embryos in large numbers (70). Ova from mice superovulated at three weeks of age or at maturity were allowed to develop to the blastocyst stage in vivo or they were cultured in vitro from the two-cell stage according to the method of Brinster (70). Even though Gates obtained an average of thirty-three blastocysts per female by culturing ova of superovulated three-week old females, ova from superovulated adult mice survived in vitro culture even more frequently (70). Thus, Gates concluded that superovulation combined with in vitro culture in Brinster's medium "promises to be a useful source of mammalian eggs in the future, especially since it is known that blastocysts from superovulated, immature mice appear to be as normal as those from spontaneously ovulated adults, both in terms of viability and in capacity to develop into fetuses of normal weight (Gates,1956)" (70).

A recent study by Spears (168) has investigated the in vitro developmental efficiency of late two-cell and four-cell ova collected from superovulated Swiss-Webster mice as compared with ova from spontaneously ovulating females of the same strain. Spears determined that approximately ninety per

cent of all cultured ova reached the blastocyst stage, and he concluded that "the viability of ova obtained from hormone treated females is equal to that of ova from spontaneous ovulation" (168).

C. STUDIES ON ENERGY REQUIREMENTS OF PREIMPLANTATION MOUSE OVA

In 1965, Brinster reported that not only lactate, but also pyruvate, oxaloacetate, and phosphoenolpyruvate (as sole energy sources in the culture medium) are able to support the in vitro development of two-cell mouse ova to the blastocyst stage (22). Of the four energy substrates, pyruvate at its optimum concentration supports the development of nearly seventy-five per cent of the two-cell ova to the blastocyst stage (22). In addition, Biggers, et al have shown that some normal fetuses and offspring can develop from superovulated two-cell mouse ova cultured to the blastocyst stage in vitro in Brinster's culture medium containing either pyruvate or lactate as the energy source (10).

The number of compounds which can be utilized as energy sources by the embryo increases as cleavage proceeds, and some compounds which will not support the development of two-cell ova in vitro (malate, citrate, acetate, glucose, and fructose) are capable of supporting the development of eight-cell embryos to the blastocyst stage in vitro (27). This suggests a possible shift in the metabolic activity of mouse embryos as development proceeds. The ability of glucose to support the development of eight-cell embryos may result from the formation of necessary enzyme systems since the uptake of glucose by the mouse embryo at the eight-cell stage is only slightly greater than at the two-cell stage (185).

The evidence suggests that there is little or no uptake of malate by the two-cell embryo but that the substrate is incorporated by active transport at later stages. Nevertheless, significant amounts of L-malate dehydrogenase are available in the zygote and two-cell embryos (26).

Radioactive tracer studies have shown that pyruvate is the best energy source for early cleavage stages of development (22,29). Nevertheless, although pyruvate, lactate and glucose are utilized equally by later pre-implantation stages, more carbon is incorporated into the embryo from glucose than pyruvate at every stage of preimplantation development. This is somewhat surprising since the two-cell embryos need pyruvate to develop into a blastocyst. It suggests that despite the incorporation of more carbon from glucose than from pyruvate into the embryo, it is the oxidation of pyruvate which is essential for development of the embryo (11,13).

Lactic acid is the main carboxylic acid which is accumulated when mouse embryos are exposed to uniformly labeled glucose (184). The zygote produces five times more lactic acid than the unfertilized egg while the two-cell stage produces 15 times more. At the blastocyst stage, pyruvate and acetate are also produced at the ratio of 90:10:1 (lactate-pyruvate-acetate) (184).

D. STUDIES ON AMINO NITROGEN REQUIREMENTS OF PREIMPLANTATION MOUSE OVA

Eight-cell mouse embryos form blastocysts when cultured in Krebs-Ringer bicarbonate saline containing glucose and bovine serum albumin (197) or when amino acids or peptides are substituted for bovine serum albumin (198).

Brinster determined that a fixed nitrogen source is essential for maximum

development of two-cell embryos to the blastocyst stage since two-cell ova will not form blastocysts in the absence of bovine serum albumin (23). Although the amino nitrogen or protein requirement can also be fulfilled by the constituent amino acids of bovine serum albumin, there is no essential amino acid since removal of any single amino acid from the culture medium does not inhibit development of two-cell mouse embryos to blastocysts in vitro.

Cystine is the only amino acid whose removal from the culture medium results in a significant decrease in the number of blastocysts which develop from two-cell embryos. While exogenous amino acids are needed to supplement endogenous nitrogen stores (23) for the development of two-cell mouse embryos, the eight-cell mouse embryo can develop into a blastocyst when there is no fixed nitrogen source in the medium. Thus, the preimplantation embryo apparently can supply most or all of its essential amino acids at this stage of development.

When amino acids are not present in the culture medium, two-cell mouse ova degenerate within 24 hours whereas if a single amino acid is present some cleavage may occur. Glutathione, as the only fixed-nitrogen source in the culture medium, will support in vitro development of two-cell mouse ova into blastocysts (32). Since this compound consists of only three amino acids (glutamic acid, cysteine, and glycine), it suggests either that the amino acid requirements of mouse embryos are relatively simple even at the two-cell stage, or that the early mouse embryo can rely to a great extent on endogenous protein sources. Brinster (28) has reported a decrease in total protein content of the in vivo developed preimplantation mouse embryo from the single

cell to the morula stage indicating that the embryo may utilize endogenous sources of protein during preimplantation development.

Gwatkin (78) has studied amino acid requirements for attachment and outgrowth of mouse blastocysts grown in vitro from the two-cell stage according to the method of Brinster (20). After blastocyst formation, the embryos were transferred to a more complex, less defined medium consisting of Eagle's basal medium (49) supplemented with 1 per cent exhaustively dialyzed fetal calf serum. He determined that five amino acids are essential for outgrowth of the blastocysts in vitro (78).

E. STUDIES ON REQUIREMENTS OF NUCLEIC ACID PRECURSORS

Although autoradiographic (90,125,126,127) and biochemical studies (51, 129,177) indicate that labeled nucleosides are incorporated by early preimplantation embryos in vitro, the addition of non-labeled nucleic acid precursors to the medium does not enhance development of two-cell mouse ova to the blastocyst stage in vitro (178). This suggests the early mouse embryo has an adequate endogenous store of nucleic acid precursors or that it is capable of synthesizing them from constituents contained within the culture medium.

F. ESSENTIAL ATMOSPHERIC AND ENVIRONMENTAL CONDITIONS

Most investigators agree that control of atmospheric and environmental conditions is extremely important in the in vitro cultivation of mammalian preimplantation embryos. Critical factors include (1) composition of the gaseous phase, (2) temperature of the gas and the environment, and (3)

humidity (31).

Although nitrogen, oxygen, and carbon dioxide are essential to the gaseous phase (31), eight-cell mouse embryos develop normally in an atmosphere of 5 per cent CO_2 in air or nitrogen but they fail to cleave in 5 per cent CO_2 in oxygen (197). Similarly, if bicarbonate is replaced by phosphate or Tris buffer in the culture medium, two-cell embryos do not form blastocysts (31), and recently it has been shown that CO_2 fixation may play an important part in early embryonic metabolism (74,186). The eight-cell mouse embryo incorporates significant amounts of carbon by fixation of CO_2 from bicarbonate in the culture medium. Thus, in addition to exerting a buffering effect in the culture medium, bicarbonate appears to be an important carbon source for the developing embryo.

A humidified atmosphere at 37°C is necessary for successful in vitro culture. At slightly higher temperatures mouse embryos fail to cleave and evaporation of the culture medium results in changes in osmotic pressure and the concentration of constituents of the culture medium (31).

G. ABILITY OF COMPLEX CULTURE MEDIA TO SUPPORT IN VITRO DEVELOPMENT

Since the formulation of Brinster's method for in vitro culture, mouse ova have been cultured in vitro in several complex, undefined media (44,126, 127,133). In one culture medium containing large proportions of serum, two-cell mouse embryos develop into blastocysts in vitro, they escape from their zonae pellucidae, and they grow out on the glass substrate (44). Although two-cell ova also develop to the blastocyst stage in vitro in Brinster's

simple medium, they do not attach to the glass substrate nor do they grow out. Instead, these embryos remain in a free-floating condition for several days (78).

Nevertheless, this does not necessarily mean that enriched culture media are more capable of supporting preimplantation development of mouse ova in vitro. For example, Cole and Paul (44) tested several different culture media (including NCTC 109, 199, F.10, Eagle's, and Waymouth's) for their ability to support development of mouse ova and blastocysts obtained from superovulated, sexually mature mice. Only Waymouth's medium supported development of any two-cell mouse ova, and even in the presence of a feeder layer of irradiated HeLa cells, only 25 to 40 per cent developed to the blastocyst stage. Brinster reported that 60 to 100 per cent of the two-cell ova cultured in his simple medium reached the late blastocyst stage of preimplantation development (20).

Modifications of Waymouth's medium (including the addition of energy and lipid sources, cofactors, vitamins, nucleic acid precursors, etc.) by Cole and Paul failed to improve development of mouse ova in the absence of a feeder layer (44). Furthermore, those ova which did develop reached the blastocyst stage 12 to 18 hours later than would be expected in vivo.

Although Mulnard (132) has modified Brinster's medium by the addition of serum (and has reported good results), it should be emphasized that while supplementary compounds such as serum, embryo extract, vitamins, etc., can be added to a simple culture medium, "the use of complex natural or undefined substances in the medium makes it very difficult to determine the exact effect of other omissions or additions to the medium" (31).

H. ULTRASTRUCTURE OF MAMMALIAN OOCYTES AND PREIMPLANTATION EMBRYOS

The first electron microscopical study of a mammalian egg was completed on the mouse ovarian oocyte by Yamada, et al (205), while Sotelo and Porter (167) and Odor (135) were the first investigators to examine the fine structure of the freshly ovulated rat oocyte.

Since these early studies, the fine structure of the fertilizing mammalian spermatozoon (4,6,80,83,174), the extrusion of rat zygotic nucleoli (176), and the behavior of gamete membranes during mammalian sperm penetration of the egg (4,6,80,83) have been examined by electron microscopy. Zamboni, et al (206) have studied the human egg at the pronuclear stage, and Longo and Anderson (111) have detailed the ultrastructural transformations leading to the establishment of the two-cell stage in the rabbit.

In addition to a comparative study (54), preimplantation blastocysts of the rabbit (84,85), armadillo (52), and rat (159) have been examined in detail by electron microscopy. Moreover, the ultrastructure of implantation in the rabbit (55,105), armadillo (53), rat (55,117,134,150), and mouse (134,148, 149,152), as well as the fine structure of blastocysts transferred to extra-uterine implantation sites (i.e., kidney, testis) have been studied (145).

Early cleavage stages of the rat have been examined by electron microscopy (95,118), and Schlafke and Enders (160) (employing improved fixation methods) have described the ultrastructural changes which occur in the cytoplasm of the rat embryo during cleavage and blastocyst formation. Anderson (2) has studied fine structural alterations of mitochondria during pre-implantation stages of development in the rabbit.

These studies have shown that a number of changes occur in both nuclear and cytoplasmic structures during cleavage and blastocyst formation in the preimplantation mammalian embryo. According to Schlafke and Enders (160), "the cytological changes reflect the total differentiation of the fertilized ovum into blastocyst cytoplasm to a much more striking extent than they do differentiation of any particular cell type". In other words, there is a greater ultrastructural difference between the cells of the blastocyst and the fertilized ovum than between the different cell types of the blastocyst.

I. ULTRASTRUCTURE OF PREIMPLANTATION DEVELOPMENTAL STAGES IN THE MOUSE

Until recently, few electron microscopical studies have been completed on preimplantation development in the mouse. Published reports include the ultrastructural comparison of superovulated (normal) and mutant mouse morulae by Calarco and Brown (35), as well as Hillman's and Tasca's ultrastructural and autoradiographic study on superovulated mouse preimplantation embryos grown to the morula stage in vitro (90). Calarco and Brown have also reported on the changes in fine structure which occur during preimplantation development of superovulated mouse embryos grown in vivo through the early blastocyst stage (81 hours post-ovulation) (36). A similar study of the early mouse blastocyst in situ is also available (147).

Although the fertilized ovum of the mouse is a highly specialized cell, preimplantation development in the mouse is marked by the appearance and elaboration of cellular organelles, inclusions, and surface specializations (36). The nucleus of the zygote contains intranuclear annulate lamellae and

several dense, spherical nucleoli which are entirely fibrillar in nature (90). Crystalloids along with cytoplasmic vesicles appear for the first time (36), and parallel arrays of curved or straight cytoplasmic inclusions (known as fibrous strands) are present throughout the cytoplasm (36). Most mitochondria present in the fertilized egg are cylindrical and they often contain intracristal vacuoles. Few ribosomes are observed at this early stage of preimplantation development (36,90).

At the two-cell stage each nucleus contains 3 to 11 spherical primary nucleoli which measure 2 to 3 microns in diameter (90).

Several cytological alterations take place at the four-cell stage. Although there are fewer nucleoli per nucleus, their diameter has increased, and they consist of a central fibrillar core with a reticulated fibrillogranular cortex (36,90). Intranuclear annulate lamellae are still present and they are associated with nucleoli (90). Cytoplasmic vesicles are numerous and crystalloids are increasingly abundant at this stage of preimplantation development. Small strands of rough endoplasmic reticulum appear and vacuolated mitochondria become more spherical. The juxtanuclear Golgi apparatus, composed of 3 to 7 stacks of cisternae, is not extensive (90).

At the eight-cell stage, some moderately differentiated nucleoli (4.5 to 6.2 microns in diameter) are present as well as one or two smaller, fibrillar nucleoli with peripheral granular zones similar to those found in cell nuclei at earlier cleavage stages (90). Cytoplasmic vesicles have increased in size but they are less numerous than at earlier stages of development (38).

Each nucleus at the morula stage contains 2 to 3 nucleoli which are almost totally differentiated into fibrillar and granular areas, and

occasionally nucleoli are associated with the inner nuclear membrane (90). Partes amorphae, nevertheless, may still be present within the nucleoli (36). Polyribosomes are more abundant and connections between the outer nuclear membrane and the proliferating granular endoplasmic reticulum have been observed (36,90). The morula (early stage) has ribosomes aligned beneath the cell membranes, but later morulae display junctional complexes in the same areas (36). Cytoplasmic vesicles continue to decrease in number but crystalloids are more numerous, and they are often associated with rough endoplasmic reticulum (36,90). At the same time, microvilli proliferate along cell surfaces and intercellular spaces begin to appear between the blastomeres of the cleaving embryo (36).

At the early blastocyst stage (81 hours post-ovulation), nucleoli become more elongate and irregular, but they retain a reticular appearance and partes amorphae may still be present (36). Intranuclear annulate lamellae also may still be present (36). Cytoplasmic vesicles, though few in number, are large and they are positioned near cell borders (36). The amount of fibrous material reportedly diminishes from the early cleavage to the blastocyst stages (54). Calarco and Brown (36) claim that the early blastocyst contains relatively large amounts of polyribosomes and rough endoplasmic reticulum.

Glycogen has been detected in armadillo (52) and rat (159) blastocysts by electron microscopy. Large stores of glycogen have also been detected histochemically in mouse preimplantation stages up to the morula stage (181). However, there is a decrease in glycogen content during blastocyst formation and by the late blastocyst stage it is present in small amounts in the inner

mass cells alone. Stern and Biggers (171), employing an ultramicro method for the enzymatic determination of glycogen, reported that glycogen concentration increased sixteen-fold from the fertilized egg to the eight-cell stage, but the substance declined by twenty per cent at the blastocyst stage. Nevertheless, glycogen has not been detected by electron microscopy in any of the preimplantation stages of murine development (36,90).

Large amounts of lipid have been reported in mink and ferret blastocysts (54), and Potts (147) reported lipid droplets, up to 0.75 microns in diameter, in the early mouse blastocyst.

III. MATERIALS AND METHODS

A. ANIMAL SUBJECT

The animal chosen for this investigation was the Swiss strain of the mouse, Mus musculus.

B. CARE OF THE ANIMALS

Male and female mice were caged separately in the same room with controlled light and temperature conditions. The daily light cycle was regulated to allow sixteen hours of illumination beginning at 6:00 A.M., and room temperature was maintained at 72° F. The animals were fed a standard mouse diet (Wayne) and they drank tap water.

C. SUPEROVULATION AND MATING

For all experiments, superovulation was induced in sexually mature female mice according to the method of Brinster (20,21). Brinster determined that "the system of injections which gave the best results was 10 to 15 International Units of pregnant mare serum (PMS) (Gestyl, Organon) in 0.25 ml of 0.9% NaCl injected intraperitoneally followed 40 to 48 hours later by 10 to 15 International Units of human chorionic gonadotrophin (HCG) (Pregnyl, Organon) in 0.25 ml of 0.9% NaCl injected intraperitoneally" (21). However, in order to be as consistent as possible, all female mice used in this study were injected intraperitoneally with ten international units of pregnant mare

serum (PMS,Gestyl,Organon) in 0.25 ml of 0.9 per cent NaCl at 4:00 P.M. on the first day of each experiment. Forty-eight hours later each female was injected intraperitoneally with ten international units of human chorionic gonadotropin (HCG,Pregnyl,Organon) in 0.25 ml of 0.9 per cent NaCl.

Males were introduced to the females (one male to two females per cage) following the second injection. The following morning the females were examined for the presence of the vaginal plug as evidence that mating had occurred.

D. COLLECTION OF EMBRYOS

In the laboratory mouse, ovulation occurs approximately 11 to 14 hours after the injection of human chorionic gonadotropin (20,21,50). Since it is difficult to establish a "definite time interval between ovulation or fertilization and any particular stage of development" (21), the ages of all ova used in Brinster's experiments (those grown in vivo as well as those grown in vitro) were determined by the estimated time which had elapsed since ovulation (20,21). Hence, the ages of all the preimplantation embryos used in this investigation were also determined from the estimated time of ovulation, namely, 11 to 14 hours after the injection of human chorionic gonadotropin.

Ova were collected for two purposes: (1) for immediate fixation followed by ultrastructural examination, and (2) for in vitro culture. The technique of ova collection is described below.

1. OVA FIXED IMMEDIATELY

At the selected stage of preimplantation development, the inseminated females were killed by decapitation. A midventral incision was made from the xiphisternal junction to the urethral meatus, the genital tract was removed and placed in a Petri dish, physiological saline was applied to the excised tissue to prevent dessication, and the excess surrounding tissues were removed.

In order to recover late two-cell stage ova (44 to 47 hours after injection of HCG; 33 to 36 hours after ovulation), the ovaries and oviducts were bilaterally severed from the uterus at the level of the isthmus. Each ovarian capsule was removed and a blunt 30-gauge needle (connected to a 2 ml syringe containing cold fixative) was inserted into the fimbriated end of each oviduct. The ova were gently flushed with fixative (See F, page 30) from the oviduct into a depression slide which was placed immediately into a refrigerator. The same procedure was used to recover eight-cell ova (57 to 60 hours after ovulation).

For collection of older embryos (late blastocyst; 100 to 108 hours after ovulation), the uterus was ligated at the level of the cervix prior to excision of the genital tract. A 26-gauge needle was inserted into the tubal end of each uterine horn, the ligature was removed, and the embryos were flushed from the organ with cold fixative. Only the most fully expanded blastocysts were recovered for ultrastructural examination.

With regard to two-cell stage ova, 34 specimens were collected and embedded (from 9 female mice) and 13 of these were examined by electron

microscopy. In addition, 9 eight-cell mouse ova (19 specimens collected from 7 female mice) as well as 22 late mouse blastocysts (32 specimens collected from 12 female mice) were examined with the electron microscope.

2. OVA COLLECTED FOR IN VITRO CULTURE

The same basic technique was employed to recover embryos for in vitro cultivation. For these experiments, however, recovery was made under aseptic conditions and warm culture medium (37°C) (rather than fixative) was used to flush the embryos from the genital tract (20,21). Following collection in depression slides, the embryos were rinsed several times with warm culture medium and then examined with a dissecting microscope. Ova which did not appear normal or which were not at the desired stage of development were discarded.

E. IN VITRO CULTURE OF MOUSE OVA

Mouse ova were cultured in vitro according to the method of Brinster (20, 21). Fresh culture medium was prepared the evening before each experiment. Table 1 (page 26) shows the composition of the culture medium employed. The medium was sterilized by passage through a Millipore filtration apparatus (Millipore Filter # GSWP 04700; Pore Size 0.22 microns; 47 mm diameter), gassed with 5 per cent CO_2 in air, and placed in the incubator overnight (21).

The ova were cultivated in drops of sterile medium under sterile paraffin oil. The paraffin oil (white, Saybolt viscosity 125/135) was sterilized by heating in a dry oven at 120°C for 5 hours (133), and then it was equilibrated with sterile culture medium by bubbling 5 per cent CO_2 in air through a

TABLE I. Brinster's pyruvate-medium for in vitro culture of two-cell mouse embryos.

<u>Component</u>	<u>g/l</u>	<u>mM</u>
NaCl	6.978	119.39
KCl	0.356	4.78
CaCl ₂	0.189	1.71
KH ₂ PO ₄	0.162	1.19
MgSO ₄ ·7H ₂ O	0.294	1.19
NaHCO ₃	2.106	25.07
Sodium pyruvate	0.0348	0.31
Bovine serum albumin	1.000	
Penicillin	100 U/ml	
Streptomycin	50 ug/ml	

The medium is stored under 5 per cent CO₂ in air in order to maintain the pH at approximately 7.4.

Source: Thomson, J. L., and J. D. Biggers 1966 Effect of inhibitors on the development of preimplantation mouse embryos. Exp. Cell Res., 41: 411-427. (Reference # 180)

mixture of 400 ml of oil and 20 ml of sterile medium for 15 minutes (21). Subsequently, the oil was stored at 37° C under 5 per cent CO₂ in air for several days prior to use.

All instruments, glassware, rubber tubing, etc. were sterilized by heating in a dry oven for 120 minutes at 140° C or by autoclaving (133).

Preimplantation mouse embryos were obtained at several different developmental stages and they were cultured for different lengths of time. After removal from the genital tract, the embryos were rinsed, inspected, and then cultured in vitro by one of the two following methods:

(1) Ten ml of sterile paraffin oil were placed in a 60 X 15 mm Petri dish and a microdrop (50 to 100 ul) of culture medium containing 10 to 12 ova was placed beneath the oil.

(2) One ml of sterile culture medium was placed in a sterile glass depression slide. Approximately 20 ova were allowed to settle to the bottom and the entire surface of the culture medium was covered with sterile paraffin oil. The depression slide was then placed inside a sterile Petri dish.

Paraffin oil prevents evaporation of the culture medium and it aids in maintaining a sterile environment (31). Development of the embryos is similar by either method as long as paraffin oil covers the surface of the culture medium (31).

Petri dishes containing the ova were immediately transferred to a National Incubator (Model 3221-7) which maintained a humidified atmosphere of 5 per cent CO₂ in air at 37° C.

The following in vitro experiments were performed in Brinster's pyruvate-medium for in vitro culture of two-cell mouse embryos (TABLE II, page 29).

- (A) Two-cell ova were collected 33 to 36 hours after ovulation and then cultured in vitro for 72 hours.
- (B) Two-cell ova were collected 33 to 36 hours after ovulation and then cultured in vitro for 92 hours.
- (C) Ova were collected at the late morula or early blastocyst stage (79-82 hours after ovulation) and then cultured in vitro for 26 hours.
- (D) Ova were collected 104 to 107 hours after ovulation (late blastocyst stage) and then cultured in vitro for 1 hour.
- (E) Ova were collected 104 to 107 hours after ovulation (late blastocyst stage) and then cultured 1 hour in Brinster's medium containing tritiated leucine at a concentration of 5 uC/ml (DL-Leucine-4, 5-T, Nuclear-Chicago, Inc.) in vitro.
- (F) Ova were collected 104 to 107 hours after ovulation (late blastocyst stage) and then cultured in vitro for 1 hour in Brinster's culture medium containing an amount of non-labeled leucine (L-Leucine) at the same concentration as that of the radioactive leucine employed in experiment (E).

At the end of the in vitro culture period in each experiment, the Petri dishes were removed from the incubator and the developing embryos were transferred into fixative.

TABLE II.

EXPERIMENTS INVOLVING IN VITRO CULTURE OF MOUSE EMBRYOS

Experiment	Number of times Experiment Performed	Number of Embryos Cultured	Number Embryos Examined by E. M.	Culture Medium Employed	<u>Estimated Post-Ovulatory Age of Embryos (hrs)</u>		<u>Length of In Vitro Culture (hrs)</u>
					At Start of <u>In Vitro</u> Culture ¹	At Fixation	
A	5	100	17	BMOC ²	33-36	105-108	72
B	5	137	14	BMOC	33-36	125-128	92
C	5	77	13	BMOC	79-82	105-108	26
D	2	31	6	BMOC	104-107	105-108	1
E	4	83	11	BMOC + ³ H-leucine	104-107	105-108	1
F	2	37	5	BMOC + Non-labeled leucine	104-107	105-108	1

¹=Estimated; ovulation occurs approximately 11 to 14 hours after HCG injection (See Reference # 50).

²=Brinster's pyruvate-medium for in vitro culture of two-cell mouse embryos (See Reference # 180).

F. FIXATION, DEHYDRATION, AND EMBEDDING OF SPECIMENS

All non-radioactive specimens were fixed in chilled 3.15 per cent glutaraldehyde adjusted to pH 7.4 with 0.1 M phosphate buffer (157). After a one-hour fixation period, the specimens were rinsed for three hours in chilled phosphate buffer, and postfixed for thirty minutes at pH 7.4 in chilled phosphate-buffered 1 per cent OsO_4 (122).

All specimens were placed in a special microchamber (94), dehydrated in a series of chilled ethanol baths, passed through propylene oxide, and embedded in Epon 812 (112). Thin sections were cut with glass knives on a Porter-Blum MT-1 ultrathin sectioning microtome, contrast was enhanced with uranyl acetate (187) followed by lead citrate (153), and the sections were examined with an RCA EMU-3F-2 electron microscope.

G. ELECTRON MICROSCOPE AUTORADIOGRAPHY

While conditions in our laboratory were not adequate for one to undertake a full-scale autoradiographic study at the ultrastructural level, the subject was intriguing enough that a pilot study was performed on the in vivo developed "late" preimplantation mouse blastocyst.

For electron microscope autoradiography, blastocysts were collected from the uterus 104 to 107 hours after ovulation and they were then cultured in vitro in Brinster's medium containing tritiated leucine (See Section E). After one hour of incubation, the embryos were rinsed with six changes of warm (37°C) culture medium containing non-labeled leucine. Subsequently, the specimens were fixed, dehydrated, and embedded in the previously described

manner (See Section F).

Thin sections were "stained" with uranyl acetate (187), covered with a thin layer of carbon, and coated with Gevaert NUC-307 nuclear emulsion (39). Radioactive as well as emulsion-coated non-radioactive thin sections were exposed for 4 weeks in dry, light-proof boxes at 4° C. The emulsions were developed in Kodak D-19b solution for two minutes, rinsed in distilled water for thirty seconds, and fixed in 24 per cent sodium thiosulfate for two minutes. After three consecutive two-minute rinses in distilled water, the sections were stained with lead citrate (153), dried, and examined with the electron microscope.

IV. RESULTS

Prefatory Remarks

As emphasized in the statement of the problem, the primary purpose of this study was to compare the ultrastructure of the in vivo developed late preimplantation mouse blastocyst collected from the uterus 100 to 108 hours after ovulation with the fine structure of mouse blastocysts developed 72 hours in vitro from the late two-cell stage in Brinster's simple, chemically defined culture medium. Nevertheless, when it was determined that the latter blastocysts were not as differentiated ultrastructurally as those late blastocysts developed in vivo (even though both types were approximately the same post-ovulatory age), it was decided to culture blastocysts in vitro for an additional 20 hours (i.e., 92 hours beyond the late two-cell stage) in an attempt to determine whether blastocysts grown in vitro from the late two-cell stage do finally reach the state of ultrastructural differentiation which is found in late preimplantation mouse blastocysts which are developed entirely in vivo. In addition, mouse ova were allowed to develop in vivo to the late morula or early blastocyst stage (79 to 82 hours after ovulation), and then they were cultured 26 hours in vitro in order to find out if longer development within the maternal environment would improve the degree of ultrastructural differentiation at the late blastocyst stage (105 to 108 hours after ovulation).

In addition, although there are two excellent articles in the literature which describe the changes in fine structure which occur during cleavage in the mouse (36,90), two-cell and eight-cell mouse ova were also collected during

this study and examined by electron microscopy. This was done in order to more clearly understand the changes in ultrastructure which occur during mouse preimplantation development, as well as to facilitate judicious interpretation of the ultrastructural differences which exist between the in vivo and in vitro grown late preimplantation mouse blastocysts which were examined in this study.

A. OVA COLLECTED AND FIXED IMMEDIATELY

1. THE TWO-CELL MOUSE EMBRYO

(a) Nuclei

Nuclear fine structure is undifferentiated at the two-cell stage of mouse preimplantation development. Nucleoli are spherical, 2 to 3 microns in diameter, and they consist of fibrils which are tightly packed together (Figure 1). Intranuclear annulate lamellae, often seen in continuity with the inner nuclear membrane, are frequently observed, but little condensed chromatin is found in nuclei. The outer nuclear membrane is devoid of ribosomes (Figure 1).

(b) Cytoplasmic Organelles and Inclusions

At the two-cell stage, there is a relative paucity of cytoplasmic organelles. The Golgi complex is inconspicuous and mitochondria are small, vacuolated, and pleomorphic. Little, if any, rough endoplasmic reticulum is present, and ribosomes (found in groups of 3 to 5) are not numerous. Crystalloids are rarely observed but fibrous strands are abundant.

(c) Surface Specializations

Microvilli line the surface of the blastomeres but junctional complexes are not present.

2. THE EIGHT CELL-MOUSE EMBRYO

(a) Nuclei

At this stage of preimplantation development, nuclei usually contain several nucleoli with reticulated, fibrillo-granular cortices and central partes amorphae, as well as 1 to 2 primary, undifferentiated nucleoli similar to those observed at the two-cell stage. Intranuclear annulate lamellae are present and a few ribosomes line the outer membrane of each nuclear envelope.

(b) Cytoplasmic Organelles and Inclusions

There is a general increase in the number of organelles in each blastomere at the eight-cell stage. The number of ribosomes increases and a few strands of rough endoplasmic reticulum appear. Mitochondria and crystalloids increase in size and number, and fibrous strands are still numerous (Figure 2).

3. IN VIVO DEVELOPED LATE PREIMPLANTATION MOUSE BLASTOCYST

Prior to implantation, the mouse blastocyst measures 90-110 microns in diameter, displays polarity, and is covered by a single layer of trophoblast cells. At the embryonic pole, a disc-shaped group of inner mass (embryonic) cells underlies the trophoblast (Raubert's) layer while a single layer of endoderm cells separates the inner cell mass from the blastocoele. In fortuitous longitudinal sections (through the embryonic-abembryonic axis), all three cell types of the blastocyst can be observed (Figure 3). Endoderm cells are recognized by the wide channels or cisternae of rough endoplasmic reticulum (54) which occupy large areas of cytoplasm.

(A) Trophoblast and Inner Mass Cells

Trophoblast cells (Figures 3,6,16) are 7 to 12 microns in length and

SUMMARY

TABLE III. FINE STRUCTURE OF NUCLEI OF IN VIVO DEVELOPED AND IN VITRO CULTURED MOUSE BLASTOCYSTS

SPECIMEN	CONDENSED CHROMATIN	INTRANUCLEAR ANNULATE LAMELLAE	NUCLEOLI	OTHER
<u>In Vivo</u> Developed Late Blastocyst	Moderate; lines inner nuclear membrane; foci in nucleoplasm and associated with nucleolus	None observed	Well-differentiated; Consist of fibrillar and granular areas; often contact the nuclear membrane	Ribosomes line the outer nuclear membrane; perinuclear cisternae often distended and in continuity with rough endoplasmic reticulum
Blastocyst Cultured 72 hours from late two- cell stage	Very little observed lining the inner nuclear membrane, associated with nucleolus, or in nucleoplasm	Commonly observed associated with nucleoli and with inner nuclear membrane	Differ from spherical undifferentiated to moderately differentiated, rarely in contact with nuclear membrane	Limited number of ribosomes line outer nuclear membrane; perinuclear cisternae are not distended; no connections with rough endoplasmic reticulum
Blastocyst Cultured 92 hours from late two- cell stage	More heterochromatin than in 72-hour embryos; but less than in the <u>in vivo</u> developed late blastocyst	None observed	Moderately to fully differentiated in normal-appearing cells; may contact nuclear membrane	In normal-appearing cells, ribosomes line outer nuclear membrane; perinuclear cisternae may be slightly distended; few connections with rough endoplasmic reticulum observed
Blastocyst Cultured 26 hours from late morula stage	Moderate; lines inner nuclear membrane; foci in nucleoplasm and associated with nucleolus	None observed	Well-differentiated; Consist of fibrillar and granular areas; often contact the nuclear membrane	Ribosomes heavily line outer nuclear membrane; perinuclear cisternae commonly distended and occasionally in continuity with rough E. R.

SUMMARY

TABLE IV. FINE STRUCTURE OF CYTOPLASMIC ORGANELLES FOUND IN IN VIVO AND IN VITRO GROWN BLASTOCYSTS

SPECIMEN	RIBOSOMES	ROUGH ENDOPLASMIC RETICULUM	MITOCHONDRIA
<u>In Vivo</u> Developed Late Blastocyst	Numerous throughout cytoplasm of all cells.	moderate number of tubules present in cytoplasm of all cells.	Numerous; electron-dense matrix; large number of cristae per organelle; intracristal vacuoles may be present.
Blastocyst Cultured 72 hours from late two- cell stage	Appear to be less numerous than in cells of <u>in vivo</u> developed blastocysts; found in small clusters.	Appears to be less numerous than in cells of <u>in vivo</u> developed blastocysts.	fairly numerous; matrix appears somewhat less dense than in mitochondria of the <u>in vivo</u> developed late blasto- cyst; fewer cristae in some organelles; some mitochondria extremely swollen with intra- cristal vacuoles; often inti- mate association with E.R.
Blastocyst Cultured 92 hours from late two- cell stage	Appear more numerous than in 72-hour cultured embryos; less numerous than in <u>in vivo</u> developed blastocyst.	moderate number of tubules present in cytoplasm of normal- appearing cells.	Majority resemble those of the <u>in vivo</u> developed late preimplantation mouse blasto- cyst; some are extremely swollen, as in embryos cultured 72 hours <u>in vitro</u> .
Blastocyst Cultured 26 hours from late morula stage	Numerous throughout the cytoplasm of all cells.	moderate number of tubules present in cytoplasm of all cells.	Similar to mitochondria of the <u>in vivo</u> developed late preimplantation mouse blasto- cyst.

SUMMARY

TABLE IV. FINE STRUCTURE OF CYTOPLASMIC ORGANELLES FOUND IN IN VIVO AND IN VITRO GROWN BLASTOCYSTS

SPECIMEN	GOLGI COMPLEX	LYSOSOME-LIKE STRUCTURES	ENDODERM CELL DIFFERENTIATION
<u>In Vivo</u> Developed Late Blastocyst	inconspicuous; 1 to 2 stacks of 4 to 5 parallel cisternae	Commonly observed; electron-dense structures	Present
Blastocyst Cultured 72 hours from late two- cell stage	moderately distended vesicles and cisternae	Commonly observed; more flocculent, less electron- dense than in cells of the <u>in vivo</u> developed blastocyst	None observed
Blastocyst Cultured 92 hours from late two- cell stage	moderately distended vesicles and cisternae	Commonly observed in normal-appearing cells; abundant in cells which appeared auto- lytic	None observed
Blastocyst Cultured 26 hours from late morula stage	moderately distended vesicles and cisternae	Frequently present; electron-dense; similar to those found in cells of the <u>in vivo</u> developed late preimplantation blastocyst	Present

SUMMARY

TABLE V. FINE STRUCTURE OF CYTOPLASMIC INCLUSIONS FOUND IN IN VITRO AND IN VIVO GROWN BLASTOCYSTS

SPECIMEN	FIBROUS STRANDS	CYTOPLASMIC VESICLES	CRYSTALLOIDS	AUTOPHAGIC VACUOLES
<u>In Vivo</u> Developed Late Blastocyst	Rarely observed	None observed	Rarely observed	Occasionally present
Blastocyst Cultured 72 hours from late two- cell stage	Numerous	Commonly observed	Numerous	Occasionally present
Blastocyst Cultured 92 hours from late two- cell stage	Less abundant than in cells of blastocysts grown 72 hours <u>in vitro</u> ; more numerous than in cells of <u>in vivo</u> grown blastocysts	Occasionally Present	Numerous	Abundant in cell which appear to be undergoing autolysis; less common in others
Blastocyst Cultured 26 hours from late morula stage	Occasionally Present	Occasionally Present	Numerous	Occasionally present

2 to 3 microns in width. They form a continuous layer which completely surrounds the blastocyst. The inner cell mass, approximately 3 to 4 cells thick, is located at the embryonic pole. Its cells are round to oval, 6 to 10 microns in diameter, and they are separated by relatively large intercellular spaces (Figures 3,6).

(1) Nuclei

Nuclear fine structure is essentially similar in both cell types. The nuclei occupy nearly central positions in the cells, and they appear rather well-differentiated (Figures 3,5,6,7,13). The inner and outer membranes of the nuclear envelope are usually separated by wide, distended perinuclear cisternae (Figures 6,7,8,9,13,14,15). These channels sometimes are distended as much as 150 mu in width, and they contain a lightly electron-dense material (Figures 7,8,9,14). Dense, granular chromatin (heterochromatin) forms an irregular outline along the inner nuclear membranes (Figures 5,7,9,12,13), and foci of condensed chromatin also appear scattered throughout the nucleoplasm (Figures 6,9,13). Numerous ribosomes line the outer nuclear membranes (Figures 5,6,7,8,9,13,14,15), and connections with cisternae of rough endoplasmic reticulum are frequently observed (Figures 8,14).

Nucleoli are large, compact, and they appear well-differentiated in both cell types. They appear spherical to elongate (Figures 4,5,7,9,13), consist of fibrillar and granular areas (Figures 5,9), and they often approach the inner nuclear membrane (Figures 5,7). Frequently, condensed chromatin is associated with the nucleolus (Figures 5,13).

(2) Cytoplasmic Organelles

In most respects inner mass and trophoblast cells are remarkably similar

with regard to the fine structure of their cytoplasmic organelles. Ribosomes are ubiquitous (Figures 5,6,12,13) and elements of rough endoplasmic reticulum are often observed (Figures 5,6,7,8,10).

The Golgi complex, usually in juxtannuclear position, appears relatively inactive and it consists of 1 or 2 stacks of 4 to 5 flattened cisternae with slightly dilated ends (Figures 5,9,12). In some cells the Golgi complex is closely associated with rough endoplasmic reticulum and with lysosome-like structures (Figure 12).

Mitochondrial morphology varies from spherical to elongate with many organelles exhibiting intracristal vacuoles (Figures 6,10,13,16). The majority of the elongate mitochondria display cristae which are perpendicular to their longitudinal axis (Figures 7,8,16), and the mitochondrial matrix is electron-dense (Figures 6,7,8,13). In addition, matrix granules may also be observed (Figure 8).

Lysosome-like structures are relatively common throughout the cytoplasm of trophoblast and inner mass cells. These solidly electron-dense inclusions are surrounded by a single membrane and they measure 1 to 1.6 microns in diameter (Figures 10,12,16).

(3) Cytoplasmic Inclusions

Although fibrous plaques and crystalloids are numerous at earlier cleavage stages in the mouse, they are rarely observed in the late preimplantation mouse blastocyst. Autophagic vacuoles, containing cytoplasmic remnants, are occasionally observed within the cytoplasm of trophoblast and inner mass cells (Figure 11). On occasion, the apparent fusion of a lysosome-like

structure with an autophagic vacuole can be observed (Figure 16). Microtubules (Figure 7) may be seen coursing through the cytoplasm of inner mass and trophoblast cells.

(4) Surface Specializations

With regard to surface specializations microvilli can be observed on the apical (free) surface of trophoblast cells (Figures 12,13,16). They measure 1 to 2 microns in length and may contain microfilaments. On the basal (blastocelic) surface of the trophoblast cell, microvilli are smaller and less numerous. In comparison, the basal surface of the trophoblast cell has a relatively smooth contour (Figure 16).

A moderate number of caveolae, indicative of micropinocytosis, are observed on the borders of both cell types (Figures 13,16).

Junctional complexes are regularly seen at lateral borders where trophoblast cells contact one another (Figure 16). Near the apical end of lateral cell borders the cell membranes fuse to form a typical "tight junction" or zonula occludens (Figure 16) while proximally the cell membranes separate to form a zonula adherens (Figure 16). Beneath the zonula adherens, the junctional complex consists of a typical desmosome (macula adherens) (Figures 16,18), and tonofilaments are occasionally seen converging from the adjacent cytoplasm (Figure 18). Interdigitating microvilli are observed toward the basal (blastocelic) one-half to one-third of lateral trophoblast cell borders (Figures 4,16), and in some instances microvilli appear to form "tongue and groove" connections just below the junctional complex (Figure 13).

Apposition between inner mass cells, as well as between inner mass and

trophoblast cells (Figure 6) is maintained by periodically located zonulae adherentes.

The zona pellucida was not present in any embryos developed in vivo to the late blastocyst stage of preimplantation development.

(B) Endoderm Cells

The fine structure of the endoderm cell is essentially the same as the ultrastructure of trophoblast and inner mass cells with the exception that endoderm cells contain wide, branching and anastomosing channels of rough endoplasmic reticulum (Figures 19,20). Connections of these channels with perinuclear cisternae are common, and the distention of the perinuclear cisternae is usually pronounced (Figures 4,20).

(C) Electron Microscope Autoradiography

Silver grains, assumedly representing sites of incorporation of radioactive leucine, appeared over the nucleus and cytoplasm of all three cell types of the in vivo developed late preimplantation blastocyst. Most cytoplasmic grains are located over areas of free ribosomes, rough endoplasmic reticulum, and occasionally over mitochondria. Within the nucleus, grains appeared over dispersed chromatin (euchromatin) and the nucleolus (Figures 21,22). Grains were also observed over perinuclear cisternae in each cell type.

B. OVA COLLECTED FOR IN VITRO CULTURE

1. BLASTOCYSTS DEVELOPED 72 HOURS IN VITRO FROM THE LATE TWO-CELL STAGE

Blastocysts developed from ova cultivated 72 hours in vitro from the late two-cell stage (post-ovulation age approximately 105 to 108 hours) display some cytoplasmic and nuclear features which differ significantly from those encountered in the in vivo developed late preimplantation mouse blastocyst.

(A) Trophoblast and Inner Mass Cells

(1) Nuclei

Intranuclear annulate lamellae, consisting of parallel membranes separated by a 200 to 350 Å space, are commonly found in nuclei of both the trophoblast and inner mass cells (Figures 24,25,27). They may be surrounded by a thin layer of condensed chromatin (Figure 25) or they may be associated with nucleoli (Figure 27). The lamellae are often oriented either parallel or perpendicular to the nuclear envelope (Figure 24). Similar structures were not observed in nuclei of the in vivo developed blastocyst. Very little condensed chromatin is associated with the inner nuclear membrane and heterochromatin is sparse within the nucleoplasm (Figures 23,24,27). A small perinuclear cisterna exists between the inner and outer membranes of the nuclear envelope (Figures 23,24,27). Ribosomes incompletely line outer nuclear membranes (Figures 24,27) and connections of the outer nuclear membranes with tubules of rough endoplasmic reticulum were not observed.

Nucleolar appearance differs from spherical agranular (undifferentiated) (Figure 24) to moderately differentiated (Figures 23,24,27). Spherical

agranular nucleoli have a compact, fibrillar structure similar to those observed in early cleavage stages (Figure 1). Moderately differentiated nucleoli have both fibrillar and granular components, but they exhibit a more reticular appearance (Figures 23,24,27) than nucleoli found in cells of the in vivo developed blastocyst (Figure 5). Occasionally, several nucleoli in different stages of differentiation are observed within individual nuclei of inner mass cells (Figure 24). Infrequently, nucleoli are associated with the inner nuclear membrane.

(2) Cytoplasmic Organelles

Ribosomes in trophoblast and inner mass cells appear to be less numerous than in cells of the in vivo developed blastocyst, and they are clustered together in small groups (Figures 26,28). The small groups of ribosomes, which may represent polyribosomes, are interspersed between strands of fibrous material throughout the cytoplasm of both trophoblast and inner mass cells (Figures 23,24,26). Tubules of rough endoplasmic reticulum also appear to be somewhat less numerous than in cells of the in vivo developed late preimplantation blastocyst. The juxtannuclear Golgi complex is moderately enlarged and distended (Figure 30).

Mitochondria are pleomorphic although spherical and elongate one predominate. Many of the mitochondria contain intracristal vacuoles, but occasionally the organelles are extremely swollen (up to 1 micron in diameter) and contain 4 to 5 intracristal vacuoles (Figure 30). The mitochondrial matrix is relatively light, cristae differ from few to moderate in number (Figures 30,31), and arch-shaped cristae are not uncommon (Figure 30).

In many instances mitochondria are intimately associated with the endoplasmic reticulum. Long strands of endoplasmic reticulum may be associated with more than one mitochondrion, or one mitochondrion may be closely associated with more than one segment of endoplasmic reticulum. Occasionally, a fortuitous section displays a strand of endoplasmic reticulum between two mitochondria in such a manner that six membranes can be observed parallel to one another (Figure 31). The membranes of the endoplasmic reticulum may or may not retain ribosomes depending on how closely the mitochondria approach (Figures 31,32). Nevertheless, despite the close relationship, no direct continuity between the membranes of the two organelles was observed.

As opposed to the solidly electron-dense ones observed in the in vivo developed late blastocyst (Figures 12,20), lysosome-like structures in blastocysts developed 72 hours in vitro from the two-cell stage are somewhat larger (up to 2 microns in diameter) (Figure 35), and they contain patches of flocculent, electron-dense material (Figures 23,32,34,35).

(3) Cytoplasmic Inclusions

Fibrous strands and crystalloids, infrequently observed in the in vivo developed late preimplantation blastocyst, are prevalent in trophoblast and inner mass cells. Crystalloids consist of aggregates of individual units which display cross-striations at approximately 120-150 Å (Figures 24,27, 28,29,31), and they are often associated with granular endoplasmic reticulum (Figures 29,31), while fibrous strands are dispersed between small groups of ribosomes throughout the cytoplasm of both cell types (Figures 23,24,26,28, 29,35). The fibrous material is usually present as parallel arrays of curved or straight strands which exhibit cross-striations at approximately 30 mu

intervals.

Spherical cytoplasmic vesicles (0.5 to 1 micron in diameter) are prominent in inner mass (Figures 24,35) and in trophoblast cells (Figures 23, 34). They contain a central core of medium electron-dense material and are not bound or limited by a membrane. Myelin-like figures are also occasionally observed in the cytoplasm of inner mass and trophoblast cells (Figure 26).

(4) Surface Specializations

Trophoblast and inner mass cells display several types of surface specializations. Microvilli are prominent on surfaces of inner mass cells (Figure 35), but lateral trophoblast cell surfaces rarely exhibit interdigitating microvilli (Figure 34). Desmosomes are responsible for adherence of trophoblast cells to one another at their lateral borders (Figures 26,28). Although trophoblast and inner mass cells as well as inner mass cells themselves are occasionally separated by large intercellular spaces (Figure 35), they are often found in rather close apposition with their membranes running nearly parallel (Figures 23,24).

Pinocytotic vacuoles, observed on apical surfaces of trophoblast cells (Figure 33), appear to result from the fusion of long, attenuated microvillus-like structures. Caveolae, associated with micropinocytosis, were also observed on surfaces of trophoblast and inner mass cells.

Most blastocysts formed after 72 hours culture in vitro from the late two-cell stage are still surrounded by their zonae pellucidae.

(B) Endoderm Cells

In contrast to the in vivo developed late preimplantation mouse blastocyst, there was no evidence of inner mass cell differentiation into endoderm

cells in any of the blastocysts developed 72 hours in vitro.

2. BLASTOCYSTS DEVELOPED 92 HOURS IN VITRO FROM THE LATE TWO-CELL STAGE

During the additional 20 hours in culture these blastocysts undergo further expansion of the blastocoele and many escape from their zonae pellucidae.

(A) Normal-Appearing Trophoblast and Inner Mass Cells

In some aspects normal-appearing cells of blastocysts developed from late two-cell embryos for 92 hours in vitro appear more differentiated than those of embryos cultured 72 hours in vitro.

(1) Nuclei

Nuclei of normal-appearing trophoblast and inner mass cells are somewhat more differentiated than nuclei of embryos cultured 72 hours in vitro. There appears to be more condensed chromatin in the nucleoplasm, as well as along the inner nuclear membranes (Figures 36,40,41). Nucleoli contain partes fibrosae and partes granulosae (Figure 42), they are somewhat less reticulated than those seen in embryos cultured 72 hours in vitro (Figures 23,24), and they more frequently contact the inner nuclear membrane (Figure 42).

Perinuclear cisternae are somewhat distended (Figure 37) and nuclear pores are demonstrable (Figure 40). While outer nuclear membranes are usually more heavily lined with ribosomes than in embryos grown 72 hours in vitro (Compare Figures 37 and 27), relatively few connections of the outer nuclear membrane with the rough endoplasmic reticulum were observed. Intranuclear annulate lamellae, common in nuclei of 72-hour cultured embryos, are not observed in nuclei of normal-appearing cells of 92-hour cultured embryos.

(2) Cytoplasmic Organelles

Trophoblast and inner mass cells appear to contain more ribosomes than in cells of the 72-hour cultured embryos (Figures 37,38,39,40), and tubules of rough endoplasmic reticulum are also more prevalent (Figures 37,38). However, some normal-appearing cells of the inner mass group contain fewer polyribosomes than others (Figure 44).

The Golgi complex consists of moderately distended vesicles and stacks (Figure 36), and several types of lysosome-like entities are present. Solidly electron-dense lysosome-like structures (Figures 37,38) similar to those observed in cells of the in vivo developed late blastocyst are often observed, as well as lysosome-like entities similar to those found in embryos cultured 72 hours in vitro (Figure 41).

Mitochondria are pleomorphic in both cell types and they often display intracristal vacuoles (Figures 37,38,39,43). A large number of these organelles contain cristae which partially or completely traverse their longitudinal axis (Figures 36,37,39,44). The mitochondrial matrix is relatively dense and homogenous but no matrix granules are observed. The number of cristae per organelle is similar to the number found in mitochondria of the in vivo developed late blastocyst. Occasionally, extremely elongate mitochondria are observed in trophoblast cells (up to 2.5 microns) (Figure 36). On the other hand, some inner mass cell mitochondria appear extremely swollen, as in cells of embryos cultured 72 hours in vitro, and they may contain 5 to 7 intracristal vacuoles (Figure 43). One giant, swollen mitochondrion (2 microns in diameter) was observed within an apparently normal trophoblast cell (Figure 37). Mitochondria and rough endoplasmic reticulum, as in cells of

blastocysts grown 72 hours in vitro from the late two-cell stage, are often intimately associated with one another.

(3) Cytoplasmic Inclusions

Although there are more fibrous strands in inner mass (Figure 44) than in trophoblast cells (Figure 37), these inclusions are less numerous than in cells of blastocysts grown 72 hours in vitro. Crystalloids are numerous in inner mass cells where 2 or 3 of the lattice-like inclusions are often surrounded by membranes of rough endoplasmic reticulum (Figures 43,44). Microtubules are observed in trophoblast cells (Figure 36), and microfilaments line the apical surface and surround the nucleus of trophoblast cells (Figure 39).

Autophagic vacuoles (Figures 40,43) and myelin-like figures (Figure 40) are occasionally observed in the cytoplasm of normal-appearing inner mass and trophoblast cells of those embryos cultured 92 hours in vitro.

(4) Surface Specializations

Trophoblast cell surfaces appear more undulating than those of the in vivo developed late blastocyst, and they contain numerous micropinocytotic caveolae and vesicles (Figures 38,39). Microvilli appear on all surfaces, including the basal surfaces (Figures 36,39), and ribosomes may extend into them. Junctional complexes, similar to those found in late preimplantation blastocysts developed in vivo, join trophoblast cells at their lateral cell borders. Inner mass cells are separated from one another and from trophoblast cells by relatively wide intercellular spaces, but zonulae adherentes occur periodically in order to maintain cell apposition. The zona pellucida was still present in a few of the 92-hour blastocysts examined (Figures 37,

39).

(B) Abnormal-Appearing Trophoblast and Inner Mass Cells

A large number of cells in blastocysts cultured 92 hours in vitro from the two-cell stage appear to be undergoing autolysis. The majority of inner mass cells observed were abnormal-appearing. In addition, a large number of trophoblast cells also appeared abnormal. Some cells displayed lysosome-like bodies, myelin-like figures, and autophagic vacuoles but still retained intact cellular and nuclear membranes (Figure 41). Other cells displayed discontinuities in their cell membranes and contraction of cytoplasm, leaving extremely large intercellular spaces (Figure 46). Perinuclear cisternae in abnormal-appearing cells are distended (Figures 45,46) and little heterochromatin is observed in the nucleoplasm. Fibrous and granular components of nucleoli either clump together (Figure 46) or separate from one another (Figure 45) and then aggregate along the inner nuclear membrane (Figures 45, 46).

Fibrous strands are often present (Figure 45), mitochondria are distorted with only a few cristae available, and autophagic vacuoles fill the cytoplasm (Figure 45). Cells in the most advanced stages of autolysis contain few free ribosomes in their cytoplasmic matrix (Figure 47). Profiles of rough endoplasmic reticulum align parallel to one another and often exhibit foci devoid of ribosomes (Figure 47). In other areas, membranes of the endoplasmic reticulum are discontinuous leaving the ribosomes aligned (Figure 47). Often the endoplasmic reticulum becomes distended and cisternae are fragmented to form vesicular profiles which appear throughout the cytoplasm

(Figure 47). In many cells, the nuclear envelope has undergone dissolution and only clumped remnants of nucleoli remain (Figure 47).

(C) Endoderm Cells

There was no evidence of endoderm cell differentiation in any of the 92-hour cultured specimens examined.

3. BLASTOCYSTS COLLECTED AT THE LATE MORULA-EARLY BLASTOCYST STAGE AND CULTURED 26 HOURS IN VITRO

In many aspects, the fine structure of trophoblast and inner mass cells in blastocysts developed 26 hours in vitro from the late morula-early blastocyst stage is remarkably similar to the fine structure of these cells in in vivo developed late mouse blastocysts. These blastocysts were fully expanded and many had hatched from their zonae pellucidae.

(A) Trophoblast and Inner Mass Cells

(1) Nuclei

Nuclei of the trophoblast and inner mass cells appear well-differentiated. Inner and outer membranes of nuclear envelopes enclose a perinuclear cisterna (Figure 57) which sometimes is distended in a manner similar to perinuclear cisternae of the in vivo grown late mouse blastocyst (Figure 61). The cytoplasmic surface of outer nuclear membranes is usually heavily lined with ribosomes (Figures 56,61), but relatively few connections were observed with cisternae of rough endoplasmic reticulum.

As in nuclei of trophoblast and inner mass cells of the in vivo grown late preimplantation mouse blastocyst, heterochromatin is found in clumps throughout the nucleoplasm and it lines inner nuclear membranes (Figures 53, 56,57,61). Nucleolus-associated heterochromatin is found in nuclei of both

cell types (Figures 57,58,61). As in cells of the in vivo grown blastocyst, no intranuclear annulate lamellae were observed in nuclei of blastocysts cultured 26 hours in vitro from the late morula stage.

Nucleoli are well-differentiated into partes fibrosae and partes granulosa (Figures 53,57,58,61), and no partes amorphae were observed. Furthermore, nucleoli have a less reticular, more compact appearance than those of blastocysts cultured from the two-cell stage for 72 hours in vitro.

(2) Cytoplasmic Organelles

Trophoblast and inner mass cells are heavily laden with free ribosomes (Figures 48,53,56) and strands of rough endoplasmic reticulum are present in moderate numbers (Figures 48,54,59). As in cells of all other in vitro cultured blastocysts, the Golgi complex is moderately distended and consists of hypertrophied saccules and vesicles (Figures 53,55,56).

Mitochondria closely resemble those of the in vivo developed late blastocyst. Many are elongate with lamelliiform cristae (Figures 48,59), while others are spherical or ovoid and contain 1 or 2 intracristal vacuoles (Figures 48,56,59). The mitochondrial matrix is electron-dense and there are a large number of cristae per organelle. As in cells of all cultured embryos, many mitochondria exhibit an intimate relationship with elements of rough endoplasmic reticulum (Figures 53,56). Extremely swollen mitochondria with intracristal vacuoles in large numbers, similar to those observed in blastocysts cultured 72 and 92 hours in vitro from the late two-cell stage, are not formed in blastocysts cultured 26 hours in vitro from the late morula-early blastocyst stage.

Lysosome-like structures found in these blastocysts are more similar to those observed in blastocysts developed from the two-cell stage in vitro. These structures are large, solidly electron-dense bodies (Figure 53), and they are found in the cytoplasm of all cells. Although cytoplasmic vesicles are not numerous, individual vesicles occupying a relatively large amount of cytoplasm were occasionally observed (Figure 53)

(3) Cytoplasmic Inclusions

Crystalloids are numerous and they are often encompassed by membranes of the endoplasmic reticulum (Figures 53,54,56). Membranes of the endoplasmic reticulum which face the crystalline material are often devoid of ribosomes (Figure 54), while the outer membranes are heavily studded with ribonucleo-protein particles.

Fibrous strands are occasionally present in the cytoplasm of inner mass (Figure 56) and trophoblast cells (Figure 61). However, they are much less numerous than in cells of embryos cultured 72 and 92 hours in vitro from the late two-cell stage.

(4) Surface Specializations

Caveolae appear on all surfaces of both inner mass and trophoblast cells (Figures 59,61). Membranes of trophoblast and inner mass cells are found in relatively close apposition (Figures 53,56) or they may be separated by moderately large intercellular spaces (Figures 53,56). Periodically spaced zonulae adherentes maintain cell to cell contact. Similar membrane relationships occur between inner mass cells themselves (Figures 48,53,56).

Microvilli also appear periodically between adjacent inner mass cells

(Figure 48) and on the apical and basal surfaces of trophoblast cells (Figure 56). Lateral trophoblast cell borders display junctional complexes near their apical ends with microvilli extending into intercellular spaces near the basal surfaces. This structural arrangement is also observed in those late blastocysts grown in vivo.

(B) Endoderm Cells

As opposed to embryos cultured 72 and 92 hours in vitro from the late two-cell stage, endoderm cell differentiation occurs in blastocysts cultured 26 hours in vitro from the late morula stage (Figures 49,60). Endoderm cells differentiate from the inner cell mass and form a single layer of cells adjacent to the blastocoele. In some blastocysts, endoderm cells have begun to grow out along the inner surface of the trophectoderm (Figure 49).

(C) Sperm Tail Remnants

In two blastocysts cultured 26 hours in vitro, electron microscopy revealed the presence of sperm tails within apparently normal trophoblast cells (Figure 49). The sperm tails appeared to be limited by a membrane (Figures 50,51,52) and sections through neck and middle pieces revealed small, single membrane-bound vesicles within and without the limiting membrane (Figures 50,52).

Several electron-dense lysosome-like structures were located in the vicinity of one sperm tail (Figure 52), and in the region of the middle piece (Figure 52) an amorphous, electron-dense lysosome-like body apparently has fused with the sperm tail remnant to form an autolysosome. Sperm mitochondria displaying cristae surround the middle piece of the tail (Figure

52, small arrow), and they appear smaller than adjacent maternal mitochondria.

4. BLASTOCYSTS COLLECTED 104 to 107 HOURS AFTER OVULATION AND CULTURED ONE HOUR IN VITRO

These specimens were examined in order to determine whether there are any alterations in the fine structure of blastocysts developed in vivo and then cultured one hour in vitro in Brinster's medium. No differences in ultrastructure were detected due to the sudden change from in vivo to in vitro developmental conditions.

5. BLASTOCYSTS COLLECTED 104 to 107 HOURS AFTER OVULATION AND CULTURED ONE HOUR IN VITRO IN CULTURE MEDIUM CONTAINING NON-ISOTOPICALLY LABELLED LEUCINE.

These specimens were examined in order to determine whether the addition of free amino acids to the culture medium would result in changes in fine structure of the in vivo developed late blastocyst after 1 hour in vitro. No alterations in ultrastructure were observed.

V. DISCUSSION

In 1963, Brinster (20) stated that mouse ova developed 72 hours in vitro from the late two-cell stage (in his simple, chemically defined culture medium) were able to form late preimplantation mouse blastocysts. In addition, he claimed that development in vitro "occurs at the same rate as that in vivo" (20). While Brinster's interpretation was based on evidence that two-cell ova form "normal" blastocysts as determined by light microscopy, the results of the present investigation have shown that certain salient ultrastructural features of mouse blastocysts grown 72 hours in vitro (from the late two-cell stage) to the "late" blastocyst stage differ from those of the "late" preimplantation mouse blastocyst which has developed entirely in vivo. Hence, the first portion of this chapter will be concerned with a discussion of the various ultrastructural differences and similarities observed in this study between in vitro and in vivo developed blastocysts, and an attempt will be made to explain their functional significance.

A. NUCLEI

1. NUCLEOLI

Biochemical (51,129,177) and autoradiographic (90,125,126,127) studies indicate that synthesis of RNA begins during mouse preimplantation development, and synthesis of ribosomal RNA may begin as early as the late two-cell stage (204). In mammalian cells, nucleolar associated chromatin can serve as a template for synthesis of ribosomal RNA in the nucleolus (8). Ribosomal RNA first appears in the fibrillar areas of the nucleolus, it separates into smaller molecules, and subsequently it combines with protein to form the ribonucleoprotein (RNP) particles of the granular component of the nucleolus (71,98).

In this study, the electron microscope autoradiographic demonstration of silver grains over the nucleolus in cells of the in vivo developed late blastocyst cultured one hour in vitro in the presence of ^3H -leucine suggests that the amino acids are being incorporated into proteins which are structurally and functionally linked to RNA to form RNP particles (173). On the other hand, since the nucleolus has been implicated in the synthesis of histones (14), which account for approximately one-third of all nucleolar proteins (110), silver grains located over the nucleolus could represent amino acids which are being incorporated into newly formed nucleolar histones. Thus, these histones could be involved in the transcription of ribosomal RNA (110).

According to the literature, nucleoli of the early mouse blastocyst are largely differentiated into fibrillar and granular areas (36), but they still

may contain undifferentiated areas (partes amorphae), and as a rule, they manifest a more reticular appearance than nucleoli of the in vivo developed late preimplantation blastocyst and those blastocysts developed 26 hours in vitro from the late morula-early blastocyst stage which were examined in this investigation. The latter two types of blastocysts contain nucleoli which are well-differentiated, and their close association with condensed chromatin suggests that they are involved in synthesis of ribosomal RNA. Nucleoli of blastocysts cultured 72 hours in vitro from the late two-cell stage are generally less differentiated (more reticular) than those of the in vivo developed blastocyst (and those grown 26 hours in vitro from the late morula-early blastocyst stage), and even primary, undifferentiated nucleoli are sometimes observed.

Hillman and Tasca, employing electron microscope autoradiography to localize the uptake of tritiated uridine in cleaving mouse embryos, have shown that no labeling is associated with the fibrillar, undifferentiated nucleoli which are observed during the early two-cell stage of mouse pre-implantation development (90). Nevertheless, as cleavage proceeds the nucleoli begin to differentiate into reticulated fibrillo-granular cortices, and electron microscope autoradiography has shown that tritiated uridine is being incorporated only into these fibrillo-granular areas. At the morula stage, most nucleoli are largely reticulated into fibrillo-granular areas.

While the moderately differentiated nucleoli found in cells of blastocysts cultured 72 hours in vitro appear more reticulated than those observed in the in vivo developed late preimplantation blastocyst, they are most likely

functioning in ribosomal RNA synthesis since they do contain fibrillar and granular areas. However, since the degree of incorporation of tritiated uridine assumedly increases in proportion to the differentiation of the nucleolus (90), it would seem reasonable to expect a greater amount of ribosomal RNA synthesis in the larger, more compact, and more differentiated-appearing nucleoli of the in vivo developed late preimplantation blastocyst and those blastocysts grown 26 hours in vitro from the late morula-early blastocyst stage. It is also assumed that very little, if any, ribosomal RNA synthesis is taking place in the primary, undifferentiated nucleoli (Figure 24) which were sometimes observed in nuclei of blastocysts grown 72 hours in vitro from the late two-cell stage.

The close association of the nucleolus with the inner nuclear membrane in cells of the in vivo developed blastocyst may also be significant. While the mechanism by which nucleolar RNA leaves the nucleus is not lucid, a recent fine structural study of mammalian cells indicates that granular material of nucleolar origin is transformed into fine fibrils which pass through nuclear pores into the cytoplasm (130). Alternatively, nucleolar RNA may pass through the inner nuclear membrane into the perinuclear cisterna of the nuclear envelope (179). One of these possibilities may represent the mechanism by which ribosomal RNA is transferred from the nucleus to the cytoplasm of cells of those blastocysts which do not contain intranuclear annulate lamellae.

2. INTRANUCLEAR ANNULATE LAMELLAE

According to the literature, intranuclear annulate lamellae are present in

interphase nuclei of preimplantation mouse embryos, but they decrease in number as cleavage proceeds (36,90). The lamellae are often associated with nucleoli, and they appear to be continuous with the inner nuclear membrane (90). In this study, intranuclear annulate lamellae were observed only in nuclei of inner mass and trophoblast cells of embryos which were cultured 72 hours in vitro from the late two-cell stage.

Moses postulated that the nuclear membrane reforms after cell division by adherence of chromosomes to vesicular components which resemble endoplasmic reticulum (131). These vesicles then coalesce to form flattened cisternae which in turn fuse to form the nuclear membrane. It is possible that intranuclear annulate lamellae are remnants of endoplasmic reticulum which were not incorporated into the nuclear membrane, but which were trapped within the nucleus (36). If this were the case, one would expect to find approximately the same number of lamellae in all cleaving embryos. According to the literature, however, there are fewer intranuclear annulate lamellae in cells of the early blastocyst than in cells of earlier cleavage stage embryos (36).

Alternatively, Kessel (99,100) suggests that intranuclear annulate lamellae could form by the fusion of vesicles which have become detached due to blebbing activity of the inner nuclear membrane. By constriction at the base of each bleb, vesicles detach and fuse with one another to form lamellae. The membranous structures increase in length by a continuation of this process, and since the intranuclear lamellae are continuous with the inner nuclear membrane, they could form a continuous system with the nuclear envelope and the endoplasmic reticulum (37).

A similar system of intranuclear membranes in nucleoli of crayfish oocytes has been functionally related to intranucleolar transfer of ribosomal RNA molecules following their transcription on DNA of the nucleolar cores (101). Intranuclear annulate lamellae have been observed in tunicate oocytes (99) and fertilized ova of the rabbit (207), as well as in cells of sea urchin embryos (88) and in adult somatic cells (77,79,96). Hence, the structures are present in young and undifferentiated cells, in rapidly proliferating cells, and in cells of normal metabolic activity.

Kessel has suggested that intranuclear annulate lamellae are involved in a specialized type of nuclear-cytoplasmic interaction which is related to differentiation (99,100). Although their functional significance in pre-implantation mouse embryos is not fully understood, it is possible that the lamellae function in the transfer of ribosomal RNA from the nucleus to the cytoplasm in embryos cultured 72 hours in vitro from the late two-cell stage. Furthermore, their apparent disappearance at the late blastocyst stage of in vivo preimplantation development may indicate that an important aspect of cellular differentiation has been completed.

3. HETEROCHROMATIN AND EUCHROMATIN

While little condensed chromatin was observed in nuclei of blastocysts cultured in vitro for 72 hours, there appeared to be a slight increase in heterochromatin in normal-appearing cells of embryos cultured 92 hours in vitro. However, the greatest amount of heterochromatin was observed in nuclei of cells of the in vivo developed late preimplantation blastocyst, and

in nuclei of cells of embryos grown 26 hours in vitro from the late morula-early blastocyst stage.

Condensed chromatin or heterochromatin is considered to be the morphological manifestation of the suppression of gene activity (34). Genes located within euchromatin (dispersed chromatin), on the other hand, are believed to be involved in the synthesis of m-RNA (65). It has been shown that polycationic histone proteins may repress DNA template functioning by stabilizing DNA double helices against strand separation (66). Therefore, histones may be involved in the regulation of the genetic activity of the DNA to which they are complexed. Recent in vitro findings in bacteria suggest that histones may indirectly influence RNA synthesis by interacting with RNA polymerase for the reversible inactivation of the enzyme (169,170).

In this study, autoradiography coupled with electron microscopy has shown that developed silver grains are located over the nucleoplasm (especially over euchromatin), and they may result from radioactive amino acids which are being incorporated into histones involved in the transcription of messenger RNA.

Evidence derived from light microscope autoradiography indicates that heterochromatin formation may begin as early as the four-cell stage of mouse preimplantation development (64). In this study, the observations of moderate amounts of heterochromatin lining the inner nuclear membrane and dispersed throughout the nucleoplasm of cells of in vivo developed blastocysts and blastocysts grown 26 hours in vitro (from the late two-cell stage) tend to support the concept that "heterochromatization of chromosomal segments is

already instrumental in directing and controlling differentiation of development in the mouse" during early preimplantation development (64). On the other hand, the very limited amounts of condensed chromatin found in nuclei in embryos cultured in vitro from the two-cell stage for 72 hours may represent another ultrastructural indication that these embryos are somewhat less differentiated than those which are developed in vivo to the late blastocyst stage.

B. CYTOPLASMIC ORGANELLES

1. RIBOSOMES, ROUGH ENDOPLASMIC RETICULUM, AND PROTEIN SYNTHESIS

In general, large numbers of free ribosomes are characteristic of rapidly proliferating embryonic cells which synthesize protein for growth, while differentiated cells tend to have fewer ribosomes but more elements of rough endoplasmic reticulum. According to the literature, however, there is a general increase in both the number of ribosomes as well as the amount of rough endoplasmic reticulum during preimplantation development in the mouse (36,54,90).

This study has demonstrated that free ribosomes are abundant in both trophoblast and inner mass cells of the in vivo grown late mouse blastocyst, and there is little difference in the amount of rough endoplasmic reticulum between the two cell types. The ultrastructural observations of large numbers of free ribosomes, the connections between channels of the rough endoplasmic reticulum and the ribosome studded outer nuclear membranes, as well as the high resolution autoradiographic indication that amino acids are incorporated into all cells of the in vivo developed late preimplantation mouse blastocyst

support Weitlauf's claim (192,193,194,195) that the mouse embryo is actively engaged in protein synthesis immediately prior to implantation.

The wide, interconnecting cisternae of rough endoplasmic reticulum observed in endoderm cells (in this study) are believed to represent one of the first ultrastructural indications of cell differentiation amongst the cells of the late preimplantation mouse blastocyst (54), and the amorphous, finely granular material found within the cisternae may represent a proteinaceous product. If this is the case, endoderm cells may be similar to plasma cells which store proteinaceous products within the endoplasmic reticulum itself, rather than concentrating the product into secretory granules in the Golgi area (59).

Ribosomes and tubules of rough endoplasmic reticulum appear to be less numerous in cells of mouse embryos cultured 72 hours in vitro from the late two-cell stage than in cells of the in vivo developed late preimplantation mouse blastocyst. Although one cannot make dogmatic statements with regard to cell function on the basis of ultrastructural evidence alone, from the observations in this study it is interesting to speculate that less protein synthesis is occurring in cells of the blastocysts grown 72 hours in vitro than in cells of blastocysts developed in vivo to the late blastocyst stage. Although normal-appearing cells of embryos cultured 92 hours in vitro from the late two-cell stage appear to contain more ribosomes and elements of rough endoplasmic reticulum than cells of embryos cultured 72 hours in vitro, a large number of cells in these blastocysts appear to be undergoing autolysis. In addition, neither of these cultured blastocysts (72 and 92 hours in vitro)

was observed to differentiate a layer of endoderm cells.

In comparison, embryos grown in vivo to the late morula or early blastocyst stage and then cultured 26 hours in vitro contain many ultrastructural features which appear similar to the in vivo developed late preimplantation mouse blastocyst. With regard to the number of free ribosomes and channels of rough endoplasmic reticulum, cells of these blastocysts seem to be as well differentiated as those of the in vivo developed late blastocyst. Furthermore, these blastocysts are able to differentiate a layer of endoderm cells.

2. THE GOLGI COMPLEX

Cells with secretory functions usually display an elaborate Golgi complex (38). However, since the organelle appears relatively quiescent in the in vivo developed late preimplantation mouse blastocyst, it probably does not have a major secretory role. The functional significance of the occasional close association of the Golgi complex with the rough endoplasmic reticulum is not known, nor is the significance of the moderate distension of the vesicles and saccules of the Golgi complex found in cells of cultured embryos fully understood.

3. THE MITOCHONDRIA

(A) Cristae

There appear to be more cristae per mitochondrion in the in vivo developed late preimplantation mouse blastocysts observed in this study than have been reported by other investigators in earlier cleavage stages (36,54,81,90), and mitochondria in embryos cultured 26 hours in vitro from the late morula-

early blastocyst stage are basically similar to those found in the in vivo developed blastocysts. Embryos cultured 72 hours in vitro from the late two-cell stage also contain some mitochondria similar to those found in the in vivo developed blastocyst, but they also contain mitochondria which have relatively few cristae. Greatly swollen mitochondria containing multiple intracristal vacuoles are also found in embryos cultured 72 and 92 hours in vitro.

Cristae function to increase the surface area of mitochondrial membranes which contain enzymes for electron transport and oxidative phosphorylation (143), while enzymes concerned with protein and lipid synthesis, as well as those of the Krebs cycle are located in the mitochondrial matrix (143). It has been suggested that enzymes of the respiratory chain may account for 25 per cent of the proteins found in mitochondrial membranes (106).

The metabolic rate of preimplantation mouse embryos, based on oxygen consumption, has been measured by Brinster and Mills (124). These investigators found that oxygen consumption does not change at fertilization but that it rises sharply at the eight-cell stage. Correlative electron microscopical studies have shown that two-cell and four-cell mouse embryos contain mitochondria with relatively few cristae, but at the eight-cell stage large numbers of elongate mitochondria (with more numerous cristae) appear. These changes in mitochondrial morphology probably represent structural manifestations of the increased oxygen consumption which takes place at this stage of development (90).

The increase in oxygen consumption rate continues until the blastocyst

stage (108 hours post-ovulation) when the embryos have an oxygen consumption rate which is similar to that of brain tissue (123). The large number of cristae found in blastocysts cultured 26 hours in vitro from the late morula stage, as well as in blastocysts developed in vivo correlate well with the increased oxygen utilization by mouse embryos which has been shown to occur during the later stages of mouse preimplantation development. Since some mitochondria of embryos grown 72 hours in vitro from the late two-cell stage contain fewer cristae and a less dense matrix, these appear to be more ultra-structural indications that these embryos are less differentiated than the in vivo developed late preimplantation mouse blastocyst.

The theory that the number of cristae reflects the function of the mitochondrion is supported by observations in rabbit preimplantation embryos. Fridhandler, et al (68) found no significant increase in oxygen uptake from fertilization until the morula stage, but at the blastocyst stage there was a sudden increase in oxygen consumption. In support of this, Anderson, et al have observed an increase in the number of cristae per mitochondrion (as well as an increase in the number of mitochondria) at the early blastocyst stage of rabbit preimplantation development (2).

(B) Mitochondria and Rough Endoplasmic Reticulum

An intimate association between mitochondria and the rough endoplasmic reticulum is observed in all cultured embryos. Mitochondria are occasionally located in proximity to the granular endoplasmic reticulum in cells of the in vivo developed blastocyst, but their association was never as close as that observed in cells of the cultured embryos. Similar associations have been observed in oocytes of the hamster (189), guinea pig (1), and mouse (156).

Direct connections between outer mitochondrial membranes and those of the rough endoplasmic reticulum (which establish continuity between cisternae of rough endoplasmic reticulum and the intracristal spaces of the mitochondrion) have been observed in mouse oocytes (156) and in liver cells (72). Such connections have been related to (i) the involvement of cytoplasmic membranes in mitochondrial genesis (3,154), and (ii) the formation of a channel for the transfer of substances to and from the mitochondrion (17). Biochemical (86, 97) and autoradiographic (48,161) studies have shown that enzymes are first synthesized on the rough endoplasmic reticulum and then they are transferred to their site of action in the mitochondrion. Since mitochondria can only synthesize insoluble structural proteins (155,183), the localization of silver grains over the mitochondria in the autoradiographic study reported in this investigation suggests either local synthesis or continual turnover of protein in these organelles (7).

Close juxtaposition of membranes of rough endoplasmic reticulum and mitochondria, as observed in cells of all the in vitro cultured blastocysts examined in this study, may also function in the transfer of materials between the organelles. This could take place in the form of active transport of molecules from the reticulum into the mitochondrion (72).

On the other hand, the intimate morphological association of the two organelles found in in vitro cultured embryos could represent a local site of energy utilization since mitochondrial distribution within the cytoplasm of cells is often related to their function as suppliers of energy. In some cells they are localized in regions where energy is needed. For example, the close association of mitochondria with the longitudinal contractile fibers

of the mammalian spermatozoon is believed to be a structural pattern which insures a short diffusion path for ATP needed for sperm motility (59). In other cells, however, mitochondria are free to move and carry ATP to sites where it is needed. The close proximity of mitochondria and the endoplasmic reticulum to one another in cells of the in vitro cultured embryos may insure a relatively short pathway for the transfer of ATP needed for protein synthesis during development. Hence, it is possible that the less than perfect conditions of in vitro culture force these morphological relationships which were not observed in in vivo developed late preimplantation blastocysts.

(C) Mitochondrial Granules

Mitochondrial granules have been described as sites for the binding of bivalent ions such as calcium and magnesium (144). Why these granules do not appear in mitochondria of the in vitro cultured blastocysts is not known. However, there may be an excess of these ions in the culture medium and storage may not be necessary.

(D) Vacuolated Mitochondria

Vacuolated mitochondria, similar to those observed in all blastocysts observed in this study, have also been observed by other investigators in normal mouse ovarian oocytes (137,138,203,205) and in preimplantation mouse embryos (36,90,158). Intracristal vacuoles have been related to normal structural adaptations of mitochondria to changing physiological requirements of oocytes (203), and to mitochondriogenesis (2). Mitochondria containing 1 to 2 intracristal vacuoles are considered normal cellular organelles of preimplantation mouse embryos (36,90).

(E) Mitochondrial Swelling

Salts of the culture medium used in this investigation resemble ions contained in blood serum (31). While the osmolarity of the culture medium employed, the osmolarity of Fallopian tube secretions, and the osmolarity of normal blood are nearly the same (0.308 osmols) (31), two-cell mouse ova are able to develop into blastocysts in culture media with osmolar concentrations ranging from 0.200 to 0.354 (21). However, the optimum osmolarity for in vitro development of mouse embryos (0.276 osmols) (21) is similar to that of media used for in vitro culture of HeLa cells (49) and lymphocytes (182).

While the reasons for differences in optimum ionic concentration in vitro and in vivo is not known, Stubblefield and Mueller (172) suggest that the balance between intracellular and extracellular ions may be controlled by hormonal mediation of ionic transport systems. Since in vitro culture conditions do not duplicate maternal conditions, embryonic cells may have different osmolar requirements in vitro than when developing under in vivo conditions. Nevertheless, since normal-appearing mitochondria are also present in the cytoplasm of cells which contain swollen mitochondria, it would appear that differences in osmolarity are not key factors. Moreover, mitochondria observed in embryos cultured 26 hours in vitro from the late morula-early blastocyst stage appear similar to those of the in vivo developed late preimplantation blastocyst.

What then is the functional significance, if any, of the swollen mitochondria? Packer has shown that liver and kidney mitochondria undergo a 2 to 3-fold reversible volume change in vitro. Swelling occurs during normal function of the respiratory chain (141), and isolated mitochondria can undergo reversible changes in size during transitions between different metabolic

stages (140). Under conditions favorable for oxidative phosphorylation (in isolated mitochondria), both processes can be regulated by the intramitochondrial ADP concentration. Metabolic conditions which increase the intramitochondrial ADP concentrations favor shrinking, whereas in the absence of ADP or in the presence of oxidizable substrates, swelling ensues in a manner which appears directly related to the rate of electron transport. In the absence of a phosphate acceptor, mitochondria swell and they take up water and solutes. As this process occurs, the membranes of the cristae separate and the intracristal space enlarges.

Therefore, swollen mitochondria with numerous intracristal vacuoles observed in cells of embryos cultured 72 and 92 hours in vitro from the late two-cell stage may result from insufficient concentrations of ADP due to leakage of the phosphate acceptor from the blastomeres into the culture medium. On the other hand, this condition could also result from an excess of oxidizable pyruvate in the culture medium at this stage of in vitro development, especially since there is evidence that mouse embryos rely less on Krebs cycle activity and more on Embden-Meyerof anaerobic pathways for energy during the latter stages of preimplantation development (24,25,29,30, 123,181,185).

C. CYTOPLASMIC INCLUSIONS

1. FIBROUS MATERIAL

Although Enders and Schlafke (54) reported that the amount of fibrous material decreases during preimplantation development, Calarco and Brown (36) did not observe a diminution in the number of fibrous strands through the

early blastocyst stage. This study has shown that fibrous strands are numerous in trophoblast and inner mass cells of blastocysts formed from embryos grown 72 hours in vitro from the late two-cell stage. These inclusions are less abundant in embryos cultured 92 hours in vitro from the late two-cell stage, they are occasionally seen in cells of embryos developed 26 hours in vitro from the late morula-early blastocyst stage, but they are rarely observed in cells of the in vivo developed late preimplantation mouse blastocyst.

The formation of similar cytoplasmic inclusions has been described in rat (175) and hamster (82,175,188,189) oocytes. In hamster oocytes the fibrous strands are preserved by fixation in glutaraldehyde, but they are labile to pepsin digestion (190). This indicates that the fibrous inclusions are largely protein in consistency.

With regard to their function, Szollosi (175) suggests the fibrous material may represent a "yolky substance". On the other hand, Weakley claims that the fibrous elements may represent sites for enzyme location (190) or that they may serve as a "temporary" endoplasmic reticulum during cleavage (190). Since the strands are rarely observed in the in vivo developed late blastocyst, the material may be utilized under in vivo conditions as an endogenous source of protein during late preimplantation development. This view is favored since Brinster (28) has shown that there is a decrease in the amount of protein within the cleaving embryos from the unfertilized egg to the late morula stage. Moreover, eight-cell mouse embryos will cleave in a medium devoid of a nitrogen source, whereas two-cell mouse embryos will not cleave under similar conditions (27). This suggests that the developing

embryos can utilize endogenous protein sources during later preimplantation stages. Alternatively, the disappearance of the fibrous material at the stage when free ribosomes and rough endoplasmic reticulum are proliferating does not rule out the possibility that they have a function which is related to protein synthesis during early cleavage.

2. CRYSTALLOIDS

According to several investigators, crystalloids are normally present in all preimplantation stages (35,36,54,90) but they are most numerous in the early mouse blastocyst (36,54,90). Nevertheless, these lattice-like inclusions are rarely observed in cells of the late blastocysts developed in vivo which were examined by electron microscopy during this investigation.

As with the above described material (fibrous material), the function of the crystalline material is not known. Calarco and Brown (36) have related crystalloids to the "dark yolk" described by Lewis and Wright (109). However, the intimate association of the crystalline material with the rough endoplasmic reticulum suggests the inclusions may be protein in nature and that they may be synthesized by the granular membranous system. In some types of vertebrate cells, crystalline material is both synthesized by and stored within the endoplasmic reticulum (59).

Although Calarco and Brown (36) did not detect a decrease in the number of crystalloids during preimplantation development up to the early blastocyst stage, Enders and Schlafke (54) reported fewer crystalloids in late blastocysts of several mammals. In this study, crystalloids were seldom observed in cells

of the "late" mouse blastocyst developed in vivo, and their apparent rapid dissolution suggests the material represents stored protein which is utilized by the embryo immediately prior to implantation. The persistence of the inclusions in cells of all in vitro cultured embryos may represent another aspect of delayed differentiation due to development of these embryos outside the maternal environment.

3. CYTOPLASMIC VESICLES

Cytoplasmic vesicles are spherical entities which contain a medium electron-dense material but which are not limited by a membrane (35,36). Calarco and Brown (35,36) have related these vesicles to the "light yolk globules" described by Lewis and Wright (109). They also resemble lipid-like inclusions found in preimplantation embryos of several mammalian species (54).

Blastocele formation and expansion is most likely due to ionic variation of the fluid within the blastocyst cavity and the surrounding environment. There is a large increase in the wet weight of the rabbit blastocyst between the fifth and sixth days after mating due to increased uptake of water (113), and Brinster's demonstration of a potential difference across the trophoblast of the rabbit blastocyst supports the concept that some components of the fluid surrounding the developing blastocyst are actively transported across the trophoblast in order to accumulate water by the process of osmosis with subsequent expansion of the blastocele (45).

Morphological evidence indicates that the cytoplasmic vesicles may be involved in formation of the blastocele (36,121). While continuity with the

cell membrane has not been observed, the location of cytoplasmic vesicles near membranes of the blastomeres lead Calarco and Brown to suggest that the vesicles may release their contents into the intercellular spaces and thereby contribute to formation of the blastocele (36).

According to Calarco and Brown (36), cytoplasmic vesicles are rarely observed in the early mouse blastocyst (36). In this study, cytoplasmic vesicles were not observed in the "late" mouse blastocyst developed in vivo, and only rarely were the vesicles found in embryos cultured 92 hours in vitro from the late two-cell stage or in embryos cultured 26 hours in vitro from the late morula-early blastocyst stage. If cytoplasmic vesicles are involved in blastocele formation, this would indicate that blastocyst expansion is relatively complete in these embryos. On the other hand, large numbers of these vesicles were observed in embryos cultured 72 hours in vitro (from the late two-cell stage) suggesting that these embryos are not fully expanded.

D. SURFACE SPECIALIZATIONS

1. JUNCTIONAL COMPLEXES

Junctional complexes appear at the eight-cell stage in the rat (160) and at the morula stage in the mouse (36). According to Enders and Schlafke (54), the blastocyst stage can be considered to begin when junctional complexes have formed between the blastomeres. Calarco and Brown (35,36) have shown that large numbers of ribosomes are aligned immediately beneath blastomere membranes in four-cell and eight-cell mouse embryos and they suggest these ribosomes are involved in synthesis of the proteinaceous components of the junctional

complexes.

Schlafke and Enders (160) have proposed that junctional complexes may function to electrically couple blastomeres of the cleaving mammalian embryo as they do during cleavage of newt (93) and squid (146) embryos. In addition to maintaining cohesion of the blastomeres, junctional complexes may be involved in retention of fluid in the blastocoele since they appear just prior to blastocoele formation (35,36). These surface specializations may play the role of a diffusion barrier by sealing the intercellular space between trophoblast cells since there is evidence that intercellular material cannot pass through junctional complexes in epithelial cells (57).

2. MICROVILLI

The usual interpretation of the functional significance of microvilli is that they increase the area of the cell surface which is exposed to materials which are to be absorbed (i.e., ions and substances of low molecular weight) (59). In ovarian follicles, oocyte microvilli penetrate the surrounding zona pellucida to intermingle with follicle cell processes from which they presumably derive their nutrition (136,137,138). During cleavage in the rat, the microvilli withdraw from the zona pellucida and become less numerous (16) while the surrounding granulosa or follicle cells degenerate (15).

Few microvilli are found on the surface of the unfertilized mouse egg, but they become more numerous in later preimplantation stages (36). Calarco and Brown (36) suggest that as early as the four-cell to eight-cell stages some microvilli function in the adherence of blastomeres. The "tongue and groove" structural arrangement observed between lateral borders of trophoblast cells

in the in vivo developed blastocysts examined in this study may function in a similar manner.

3. PINOCYTOSIS AND MICROPINOCYTOSIS

Pinocytosis was originally described by Lewis (108) who studied living cells in tissue culture by phase contrast microscopy. Lewis described the uptake of fluids by active movements of undulating membranes which appeared to arise at the cell periphery. Extending upward from the free surface, the membranes entrapped fluid into vesicles. Later, the vesicles (more than one micron in diameter) moved into the interior of the cell and became progressively smaller.

Later studies suggested that pinocytosis might be important for cellular uptake of proteins. Electron microscopy of capillary endothelial cells has shown that projections or flaps are present at the free margins of these cells undergoing pinocytosis. These flaps are usually about 80 mu thick and they impound material by curving over and coalescing with the plasma membrane (46,60). Assumedly, these are the large vesicles originally reported by Lewis. Hence, the term pinocytosis was first applied to entrapment of fluid by projecting folds and evidence for this type of pinocytosis was demonstrated in embryos cultured 72 hours in vitro from the two-cell stage.

Electron microscopical studies of endothelial cells have also revealed large numbers of 650-800 Å diameter micropinocytotic vesicles which appear to invaginate from the cell membrane (142). After detaching from the luminal surface, these vesicles migrate across the cell and discharge their contents at the basal surface of capillary endothelial cells.

The use of electron-opaque markers such as ferritin, an iron-protein structural complex with a molecular weight of 465,000 to 480,000 (89) and a diameter of 110 Å (43), has established the concept that pinocytosis is involved in the active transport of not only fluids but macromolecules as well. Ferritin molecules are first absorbed to the cell surface and then invagination takes place forming open pits on the luminal surface. Vesicles then detach from the membrane and form intracytoplasmic vesicles which finally dissolve and release ferritin into the cytoplasm (83). Therefore, micropinocytosis appears to be a structural manifestation of the method by which solutes and macromolecules are transported in mass across the surface membrane.

Although Calarco and Brown (36) reported little micropinocytotic activity during mouse preimplantation development, this study has demonstrated a substantial amount of micropinocytotic activity in the in vivo developed late mouse blastocyst and in cells of the in vitro cultured embryos. Micropinocytotic caveolae are seen in blastocysts of several species (54), and Hadek (83) has shown the uptake of ferritin by micropinocytosis in the unfertilized egg and preimplantation stages of the rabbit.

Pinocytosis is responsive to external stimulus (41) and substances which can induce pinocytosis include amino acids, proteins, and simple salts (116). In all cases, the inducing agents are positively charged particles (cations). It is assumed that bovine serum albumin, with a molecular weight of approximately 69,000, is able to penetrate the zona pellucida of the mouse egg (73), and this protein and salts found in the culture medium stimulate micropinocytosis in in vitro cultured embryos while in the in vivo developed

blastocysts the phenomenon may reflect increased uptake of nutrient materials and free amino acids which are available within the uterus just prior to implantation (76).

The potential difference detected across the rabbit trophoblast suggests that micropinocytosis could also be actively involved in formation of the blastocele (45).

4. SPERM TAIL ELEMENTS IN MOUSE BLASTOCYSTS CULTURED 26 HOURS IN VITRO

This appears to be the first ultrastructural observation of sperm tails in the mouse blastocyst. Although axial filaments of the fertilizing mouse spermatozoon are present within the zygote a few hours after copulation (151), swelling and breakdown of the paternal mitochondria have been reported to occur prior to the first cleavage division, and the sperm tail disintegrates soon thereafter (151).

In addition to this, the following features also make the observations unusual: (1) the limiting membrane which surrounds the tail of the spermatozoon, and (2) the presence of intact sperm tail mitochondria at this stage of development.

A limiting membrane around the tail of the spermatozoon at this stage of development is unusual since fertilization involves fusion of the plasma membranes of both gametes (83,174). Furthermore, ultrastructural studies have shown that the mammalian sperm does not manifest a cytolemma following incorporation into the ovum (6,83,151,174).

Since the incorporated spermatozoa observed in this study are still surrounded by their cytolemma, it is doubtful that they represent remnants of

fertilizing spermatozoa. Therefore, they most likely represent supernumerary spermatozoa which were incorporated into the embryos sometime after fertilization. It is not possible to determine at which stage of development incorporation occurred, but assumedly phagocytosis of the sperm tails has been delayed as a result of the abrupt transition from in vivo to in vitro developmental conditions.

E. INFLUENCE OF THE MATERNAL ENVIRONMENT ON PREIMPLANTATION DEVELOPMENT

This portion of the discussion will attempt to offer reasonable explanations or suggestions why two-cell mouse ova cultured 72 hours in vitro to the late blastocyst stage are less differentiated in several ultrastructural aspects than those late blastocysts which have been grown entirely within the maternal reproductive tract. Moreover, suggestions will be forthcoming as to why mouse embryos developed in vivo to the late morula or early blastocyst stage and then cultured 26 hours in vitro to the late blastocyst stage are basically as well-differentiated ultrastructurally as those late preimplantation mouse blastocysts which were developed entirely in vivo.

The maternal environment is important during mammalian preimplantation development, and there are at least three stages during which changes in metabolic processes or the presence of certain maternal "factors" may be critical to the successful development of preimplantation mouse embryos: (1) the single-cell to two-cell stage, (2) the eight-cell to sixteen-cell stage, and (3) the blastocyst stage.

1. THE SINGLE-CELL TO TWO-CELL STAGE

There seems to be an important Fallopian tube "factor" since complete development of the single-cell mouse ovum to the blastocyst stage occurs only in organ cultures of the Fallopian tube in vitro (9). The fertilized ovum will develop to the early two-cell stage in vitro (202), and the late two-cell embryo will cleave to the blastocyst stage in vitro in a simple, chemically defined culture medium (20). However, early two-cell mouse embryos will not cleave unless they are cultured within the Fallopian tube (202). Assumedly, there is an unknown "factor" whose effect is important in the intermitotic period between the first and second cleavage divisions, and there is strong evidence that this factor is related to the ampullary region of the oviduct (200,201).

It should be noted, however, that Whitten and Biggers (199) have been able to grow eggs of certain hybrid mice from the single-cell fertilized stage to the blastocyst stage in vitro, and viable offspring were obtained from some of these blastocysts after their transfer to uteri of foster mothers. However, when two-cell ova from randomly bred mice were cultured simultaneously, late two-cell embryos developed into blastocysts but single-cell embryos did not.

Since the ova cultured in vitro in this study had already reached the late two-cell stage prior to their removal from the maternal environment, this particular Fallopian tube "factor" is not critical to the results observed in the present investigation.

2. THE EIGHT-CELL TO SIXTEEN-CELL STAGE

(A) Changes in Basic Metabolic Patterns

Cleaving mouse embryos are dependent on an exogenous supply of energy sources (22). Oviduct secretions in the rabbit contain pyruvate, lactate, and glucose (91), and the concentration of pyruvate found in the rabbit oviduct is similar to the optimum concentration used in Brinster's medium for in vitro cultivation of mouse embryos (22,180). Unfortunately, due to technical difficulties, similar substances have not been demonstrated in the mouse oviduct.

There is an increase in the number of compounds which will support development of mouse embryos as cleavage proceeds, and this may result from changes in cellular permeability. Malate, for example, will support development of mouse embryos beginning at the eight-cell stage, and the substrate appears to be taken up by active transport (185). The activity of malic acid dehydrogenase also increases at the eight-cell stage (26). On the other hand, the activities of lactate dehydrogenase (24) and glucose-6-phosphate dehydrogenase (25) are constant up to the eight-cell stage, but both enzymes decrease in activity thereafter.

In the rabbit, Fridhandler (67) has shown that glucose is oxidized mainly by the pentose shunt prior to blastocyst formation. Following formation of the blastocoele, oxidation is mainly by the Embden-Meyerof pathway and the tricarboxylic acid cycle. In the mouse, Brinster and Thomson (27) have demonstrated that the eight-cell embryos, but not the two-cell embryos, will develop into blastocysts in a medium which contains only glucose as an energy

source, and this may result from increased activity in the Embden-Meyerof pathway. Hexokinase, a regulatory enzyme in this pathway, increases seven-fold in activity from ovulation to implantation (30).

The increased ability of the developing embryo to utilize glucose and malate, the increased activity of malic dehydrogenase, and the increased oxygen consumption at the eight-cell stage (123) indicate that fundamental changes in embryonic energy metabolism are occurring at this stage of development. These changes occur while the developing embryos are still within the oviduct. Thus, it is possible that the oviduct exerts a positive influence or regulatory effect on the cleaving embryos during in vivo preimplantation development. Therefore, it may be more difficult for the embryos to accommodate to changes in their basic metabolic patterns (especially those which occur at the eight-cell to sixteen-cell stage) when development occurs in vitro, and as a consequence, ultrastructural differentiation is less advanced than when development occurs entirely in vivo to the late blastocyst stage of preimplantation development.

(B) Rate of Cleavage

The rate of cleavage of preimplantation embryos appears to differ among strains of mice. For example, McLaren (119) has studied the cleavage rate of mouse ova (belonging to the random-bred Q strain) in vivo and during in vitro culture from the late two-cell stage in Brinster's culture medium. While cleavage in vivo occurs approximately every 11 hours from the first division until just prior to implantation, the cleavage rate in vitro becomes progressively slower with plateaus occurring at the eight-cell and sixteen-cell

stages. On the other hand, Gates (70) has shown that developing embryos of other strains of mice (BALB/c x BALB/c; BALB/c x 129) can be identified as either fast-cleaving or slow-cleaving ones by three and one-half days after ovulation, regardless of whether development has occurred in vitro in Brinster's culture medium or entirely in vivo.

Nevertheless, Brinster (20) reported that development of ova obtained from random-bred Swiss mice (the strain also used in this study) occurs at the same rate in vitro as in vivo with cavitation appearing late on the second day of culture. In support of this, blastocysts collected in this study for ultrastructural examination after 72 hours cultivation in vitro in Brinster's medium appeared to be as fully expanded (as determined by light microscopy) as those late blastocysts which were collected after developing entirely within the maternal reproductive tract.

3. THE BLASTOCYST STAGE

(A) Uterine Factor

When oviductal embryos are transferred beneath the kidney capsule or testis, there is abundant growth of the trophoblast and extraembryonic membranes, but embryonic tissue itself does not develop (102,104). When blastocysts recovered from the uterus are transferred to these extrauterine implantation sites, approximately 25 per cent form trophoblast and embryonic tissues (12,103). However, when cleaving embryos are retained within the oviducts by ligation until the blastocyst stage, they react as oviductal stage embryos when they are transferred to extrauterine sites and no embryonic development takes place. Thus, Kirby has postulated the existence of a

uterine "factor", present in the uterus but absent from the oviduct, which is necessary for normal preimplantation development (104).

Recently, however, two-cell mouse embryos have been cultured to the blastocyst stage in vitro and then transferred to the kidney or testis (13). Some of these blastocysts form normal-appearing embryos indicating that the uterine environment may not always be essential for totipotency of the mouse egg.

(B) Loss of the Zona Pellucida

According to Orsini and McLaren (139), the majority of blastocysts in receptive, estrogen-sensitized uteri lose their zonae pellucidae by the beginning of the fifth day of pregnancy (vaginal plug=day 1) while those embryos retained in the oviduct by ligation or blastocysts in uteri of ovariectomized or lactating females retain the zona for approximately 12 hours longer. Therefore, it appears that in normal pregnancy the uterus produces an estrogen-dependent "lytic" factor which acts to weaken the zona pellucida and to expedite "hatching" of the blastocyst (139). Other investigators have shown that mouse blastocysts cultured in vitro will also "hatch" from their zonae (20,21,44) by their own unaided efforts but the process generally takes longer than it normally would in vivo (139). The above claims are supported by the ultrastructural observations reported in this study.

4. INFLUENCE OF OVARIAN HORMONES DURING PREIMPLANTATION DEVELOPMENT

Active protein synthesis in preimplantation mouse embryos, as inferred by the uptake of isotopically labeled amino acids in vivo, does not commence until the blastocyst stage (76,191). When day five blastocysts (day 1 =

vaginal plug) are transferred to oviducts of female recipients (on their second day of pregnancy) which contain two-cell ova, the blastocysts incorporate labeled amino acids at the same rate as they would in utero (192). However, the native two-cell ova exposed to the same tubal environment do not incorporate amino acids. This demonstrates that labeled amino acids are available to eggs in the oviduct as well as to those in the uterus, and it suggests that the delayed onset of protein synthesis until the blastocyst stage under in vivo conditions is due to maturation of the blastocyst rather than to a deficiency of precursors in the oviduct at earlier developmental stages.

In normal pregnancy, synergism between estrogen and progesterone begins on the fourth day (61). When mature mice are ovariectomized on the fourth day of pregnancy (day 1=vaginal plug), and in the absence of exogenous ovarian hormones, blastocysts can be recovered from the uterus as much as 36 days later (194). Exogenous hormones are necessary to induce implantation (92,164,194) and to support normal fetal development after ovariectomy (194).

Active protein synthesis in mouse preimplantation embryos in vivo begins at the late blastocyst stage (95-99 hours after ovulation), and it corresponds to the onset of the surge in estrogen secretion (61,62). Weitlauf and Greenwald have shown that in vivo incorporation of radioactive amino acids by the mouse blastocyst is also triggered by synergism of estrogen and progesterone (193), and that incorporation does not occur in the absence of either hormone (195).

During delayed implantation induced by lactation, negligible amounts of estrogen are present in the mouse until the eleventh day when hormone titers increase (75). Estrogen is responsible for the uptake of radioactive amino acids by the mouse uterine epithelium (76) and delayed implantation in the pregnant, lactating mouse results from low levels of estrogen (196).

When suckling stimulus is employed to delay implantation, the delayed implanting blastocysts do not incorporate radioactive amino acids until the ninth day of lactation (191). However, if suckling mice are removed from the mother, the delayed implanting blastocysts incorporate amino acids 24 hours later to the same extent as they normally would on day five of pregnancy (191). Amino acid incorporation also begins in delayed implanting blastocysts after estrogen is injected into the lactating mother (193). In these instances, therefore, blastocysts are exposed to estrogen prior to increased protein synthesis.

This suggests that the metabolic activity of the delayed implanting blastocyst more closely resembles the preimplantation embryo prior to blastocyst formation. In support of this, electron microscopy has revealed fine structural differences in rat blastocysts of normal pregnancy and those of delayed implantation (159). Inner mass cells of delayed implanting rat blastocysts display less granular endoplasmic reticulum in their cytoplasm, and the inner cell mass does not differentiate a layer of endoderm cells.

Gwatkin has demonstrated that blastocyst outgrowth in vitro can be controlled by the amino acid composition of the culture medium, and he suggested that outgrowth in vitro might be analogous to the early phases of

uterine implantation (78). He also suggested that selective restriction of certain amino acids from the uterus might be responsible for delayed implantation in vivo. However, Weitlauf and Greenwald (191) have shown that although blastocysts do not incorporate ^{35}S -methionine during delayed implantation, isotopically labeled amino acids are secreted into the uterus during this condition since normal day five blastocysts transferred to delayed implanting uteri are able to incorporate labeled amino acids. These results suggest that amino acids are also available in the uterine lumen, that ovarian hormones influence protein synthesis by the blastocyst more than they do the secretion of amino acids by the uterus, and that blastocysts are activated in response to the same hormonal stimulus which prepares the uterus to receive them.

When blastocysts recovered on day five of normal pregnancy are transferred to uteri of ovariectomized females, the incorporation of radioactive amino acids becomes reduced, and it finally stops after prolonged exposure (195).

It is entirely possible that ovarian hormones act directly on the pre-implantation mouse blastocyst. Smith (163) separated maternal and embryonic environments by cultivating mouse ova in vitro from the two-cell stage to the blastocyst stage in Brinster's culture medium. Thus, the embryos were not exposed to estrogen or estrogen-stimulated secretions. In order to determine whether estrogen could effect implantation, the embryos were removed from the culture medium at the blastocyst stage and transferred to ovariectomized foster mothers which had been treated with exogenous progesterone alone, or with estrogen and progesterone from the time of ovariectomy. More embryos

implanted when the foster mothers were primed with both estrogen and progesterone than when treated with progesterone alone from the time of ovariectomy. Moreover, mouse blastocysts cultured in vitro and treated with estradiol in vitro implanted in significantly higher numbers in progesterone-primed foster mothers than when untreated control blastocysts were transferred. Isotopically labeled estradiol was also taken up and bound by the blastocyst in vitro, suggesting it may act directly on the preimplantation mouse blastocyst.

Estradiol treatment of cultured blastocysts in vitro also stimulates the uptake of amino acids and they are incorporated into acid precipitable protein (163). This is strong evidence that hormonal stimulation is needed to significantly increase ribosome formation and protein synthesis in the preimplantation mouse blastocyst. Along with Schlafke's and Ender's report of less rough endoplasmic reticulum in inner mass cells and the lack of endoderm cell differentiation in the delayed implanting blastocyst of the rat (159), this study has shown that blastocysts cultured 72 hours in vitro from the late two-cell stage in Brinster's culture medium contain fewer ribosomes and less rough endoplasmic reticulum in their inner mass and trophoblast cells than are found in cells of late preimplantation mouse blastocysts developed entirely in vivo. Although embryos cultured 92 hours in vitro from the late two-cell stage undergo further changes in fine structure, they do not reach the state of ultrastructural differentiation which is characteristic of the in vivo developed late preimplantation mouse blastocyst before many of their cells become autolytic. In addition, there was no evidence of endoderm cell

differentiation in any blastocysts grown 72 or 92 hours in vitro from the late two-cell stage.

Although mouse blastocysts can incorporate amino acids in vitro in the absence of ovarian hormones (125,126,127,129), Weitlauf (195) has demonstrated that once blastocysts are activated they can continue to incorporate amino acids for another 36 hours in the absence of hormonal stimulation. Thus, amino acid incorporation in vitro by mouse blastocysts may be the result of a direct effect of ovarian hormones on the embryos prior to their recovery from the maternal environment.

As opposed to blastocysts grown 72 hours in vitro from the late two-cell stage, mouse embryos grown in vivo to the late morula or early blastocyst stage and then cultured 26 hours in vitro to the late blastocyst stage appear to contain approximately the same amount of ribosomes and rough endoplasmic reticulum in their cells as are present in the cells of the late preimplantation mouse blastocysts which have been developed entirely in vivo. Moreover, the inner cell mass of the 26-hour cultured blastocyst (as the inner cell mass of the late preimplantation mouse blastocyst developed entirely in vivo) is able to differentiate a layer of endoderm cells. In view of the above discussion, and since blastocysts cultured 26 hours in vitro from the late morula or early blastocyst stage were recovered from the maternal environment on the fourth day of pregnancy, it is possible that they were "activated" sufficiently by ovarian hormones prior to their 26-hour in vitro culture period. Hence, the results of this study support the concept that hormonal stimulation of the preimplantation mouse blastocyst may be necessary for maximum protein

synthesis and ultrastructural differentiation.

Finally, one cannot rule out the possibility that blastocysts grown 26 hours in vitro are as well-differentiated ultrastructurally as those developed entirely in vivo simply because they were allowed to complete most of their preimplantation development within the maternal reproductive tract. These blastocysts were allowed to develop in vivo to the late morula or early blastocyst stage (79 to 82 hours after ovulation). During this period of in vivo development, there are important changes in the basic metabolic patterns of the developing preimplantation mouse embryos, the embryos are exposed to several important maternal "factors", and they may be "activated" by ovarian hormones. Thus, the longer exposure to the maternal environment could account for the apparent advanced ultrastructural differentiation of mouse blastocysts grown 26 hours in vitro from the late morula or early blastocyst stage as compared with those late mouse blastocysts which were developed 72 hours in vitro from the late two-cell stage.

VI. SUMMARY AND CONCLUSIONS

The in vivo developed late preimplantation mouse blastocyst has been examined by electron microscopy and electron microscope autoradiography, and it has been compared to blastocysts grown for 72 and 92 hours in vitro from the late two-cell stage, as well as with blastocysts grown 26 hours in vitro from the late morula or early blastocyst stage. The following was observed:

1. Nuclei of trophoblast and inner mass cells of the in vivo developed late preimplantation mouse blastocyst are well-differentiated. Outer nuclear membranes, studded with ribosomes, are often seen in direct continuity with the rough endoplasmic reticulum, and perinuclear cisternae are distended with a lightly electron-dense substance. Nucleoli are also well-differentiated, they contain fibrillar and granular areas, and they often contact the inner nuclear membrane. Foci of condensed chromatin are found associated with nucleoli, in the nucleoplasm, as well as lining the inner nuclear membranes.

Trophoblast and inner mass cells of the in vivo developed late preimplantation mouse blastocyst contain numerous free ribosomes and moderate amounts of granular endoplasmic reticulum. The juxtannuclear Golgi apparatus appears relatively quiescent while mitochondria, varying from spherical to elongate, often contain intracristal vacuoles. Lysosome-like structures are found in both inner mass and trophoblast cells, but crystalloids and fibrous strands

are rarely observed.

Although microvilli are found on all cell surfaces, junctional complexes are located only at the apical ends of lateral trophoblast cell borders. Intermittent zonulae adherentes maintain a relatively loose apposition between inner mass cells and trophoblast cells, as well as between inner mass cells themselves. Caveolae are found on all cell surfaces.

Endoderm cells are differentiated from the inner cell mass in the in vivo developed late preimplantation mouse blastocyst, and they represent one of the first ultrastructural indications of differentiation amongst cells of the mouse blastocyst.

High resolution autoradiography suggests that all cells of the in vivo developed late preimplantation mouse blastocyst are actively incorporating radioactive amino acids.

2. The fine structure of mouse embryos grown 72 hours in vitro from the late two-cell stage generally appears less differentiated than that of the in vivo developed late preimplantation mouse blastocyst.

Nuclei contain annulate lamellae, which are not present in cells of the in vivo developed late preimplantation mouse blastocyst, and little condensed chromatin is present within the nuclei. Nucleolar fine structure differs from spherical agranular (undifferentiated) to moderately differentiated.

Cells of these embryos contain fewer ribosomes and fewer elements of rough endoplasmic reticulum. Swollen mitochondria, containing multiple intracristal vacuoles, are often observed in cells of embryos grown 72 hours in vitro from the late two-cell stage, and they are often intimately associated with the

rough endoplasmic reticulum. The Golgi complex is moderately distended, and lysosome-like structures and myelin-like figures, as well as numerous crystalloids and fibrous strands are present.

As opposed to the in vivo developed late preimplantation mouse blastocyst, there was no evidence of endoderm cell differentiation in embryos cultured 72 hours in vitro from the late two-cell stage.

3. Normal-appearing cells in blastocysts cultured 92 hours in vitro from the late two-cell stage contain more ribosomes and rough endoplasmic reticulum than blastocysts cultured 72 hours in vitro from the late two-cell stage. Mitochondria were often swollen, and the Golgi complex was moderately distended as in embryos cultured 72 hours in vitro. Although fibrous strands are somewhat reduced in number, crystalloids appeared to be as numerous as in cells of embryos cultured 72 hours in vitro.

Nuclei of embryos cultured 92 hours in vitro appear to be somewhat more differentiated than those of embryos cultured 72 hours in vitro from the late two-cell stage.

Many cells of blastocysts cultured 92 hours in vitro from the late two-cell stage appear to be undergoing autolysis.

As in blastocysts developed 72 hours in vitro, there was no evidence of endoderm cell differentiation from inner mass cells in embryos cultured 92 hours in vitro from the late two-cell stage.

4. The fine structure of cells of embryos developed 26 hours in vitro after having developed to the late morula or early blastocyst stage in vivo is very similar to the fine structure of cells of the in vivo developed late pre-

implantation mouse blastocyst.

Nuclei of trophoblast and inner mass cells appear well-differentiated, and they closely resemble those of the in vivo developed late preimplantation mouse blastocyst.

Trophoblast and inner mass cells appear to contain numerous ribosomes and strands of rough endoplasmic reticulum are present in moderate numbers. In addition, mitochondria are similar to those found in cells of the in vivo developed late preimplantation mouse blastocyst.

The only differences between the fine structure of these embryos and that of the in vivo developed late preimplantation mouse blastocyst is that the Golgi complex is somewhat more distended, crystalloids are numerous, and fibrous strands are occasionally observed.

As in the in vivo developed late preimplantation mouse blastocyst, inner mass cells are able to differentiate a layer of endoderm cells in blastocysts developed 26 hours in vitro from the late morula or early blastocyst stage.

The following reasons may explain the retarded fine structural differentiation observed in embryos cultured 72 hours in vitro from the late two-cell stage:

- (1) The embryos are delayed in their differentiation due to their transfer from the maternal environment to artificial in vitro conditions,

- (2) In vitro conditions do not adequately simulate the maternal conditions for development. Under in vivo developmental conditions, preimplantation embryos evince at least three stages which are involved in critical

changes in basic metabolic patterns. Since culture conditions most likely do not duplicate in vivo developmental conditions, it may be more difficult for embryos which develop in vitro to facilitate the changes in metabolic pathways which normally occur during preimplantation development than when the embryos are allowed to develop within the oviduct and uterus.

(3) Certain intrinsic "factors", normally present in the maternal environment and which effect the developing mouse embryos, do not appear to be duplicated by the simple in vitro culture system which was employed in this study.

(4) Mouse embryos developed 72 (or 92) hours in vitro from the late two-cell stage are not "activated" by ovarian hormones which may be necessary for maximum protein synthesis and ultrastructural differentiation.

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PLATE I

EXPLANATION OF FIGURES

Figure 1. Electron micrograph of a nucleus of an in vivo developed two-cell mouse embryo containing a fibrillar, undifferentiated nucleolus (N). E, nuclear envelope. X30,000.

Figure 2. Electron micrograph of a blastomere of an in vivo developed eight-cell mouse embryo. The plane of section is tangential to the nucleus (N) and nuclear pores (arrow) can be observed. Note the numerous fibrous strands (F) throughout the cytoplasm. Notice also that the mitochondria (M) contain few cristae. There is also a paucity of cytoplasmic ribosomes. L, lipid inclusion. X 22,000.

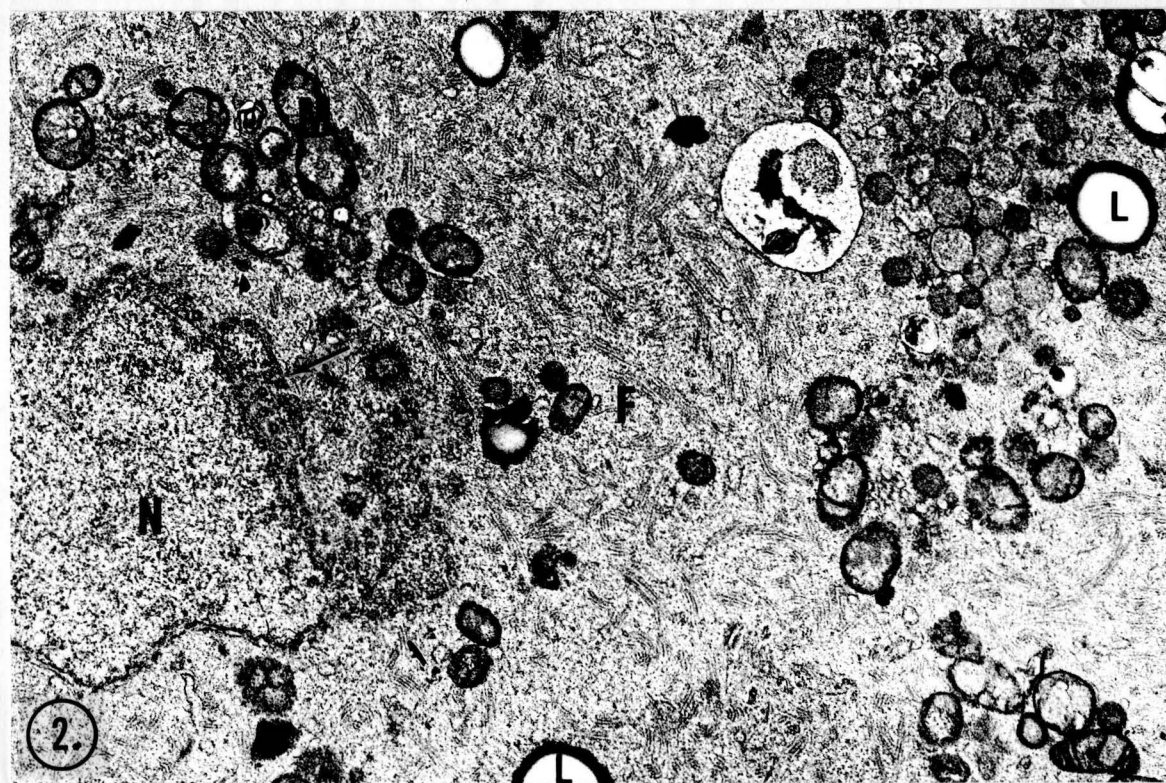
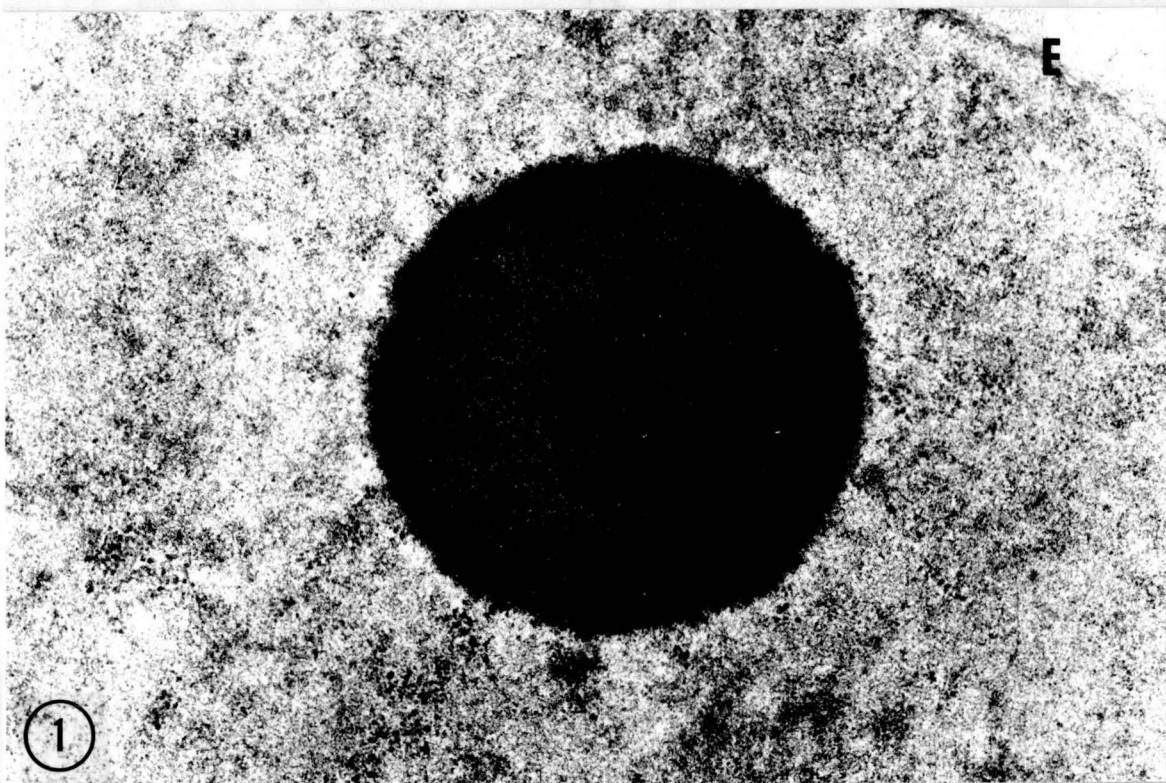


PLATE II

EXPLANATION OF FIGURE

Figure 3. A longitudinal section through an in vivo developed late pre-implantation mouse blastocyst showing trophoblast (T), inner mass (M), and endoderm (E) cells. Endoderm cells are identified by the wide channels of rough endoplasmic reticulum which are found in their cytoplasm. X4,000.

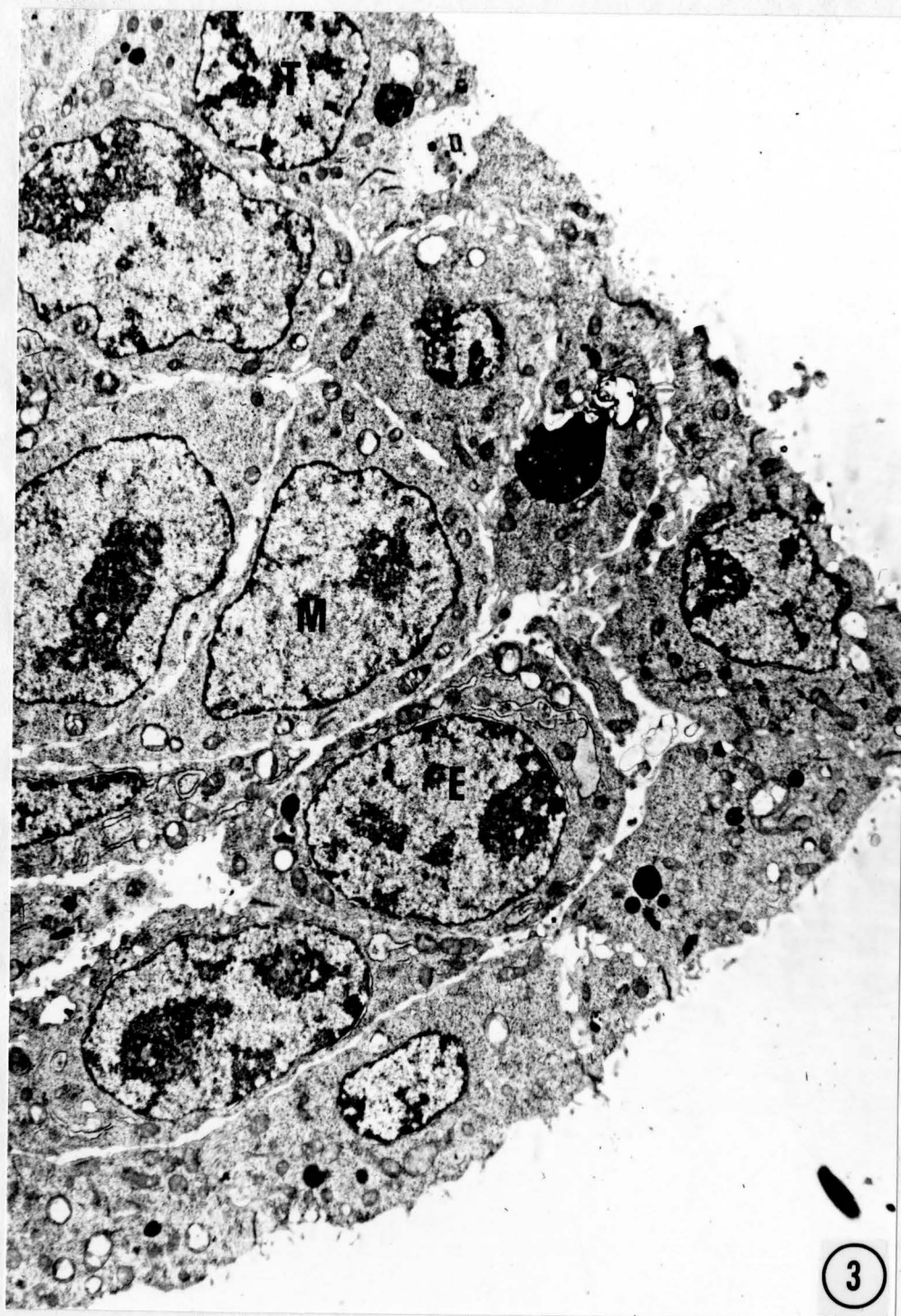
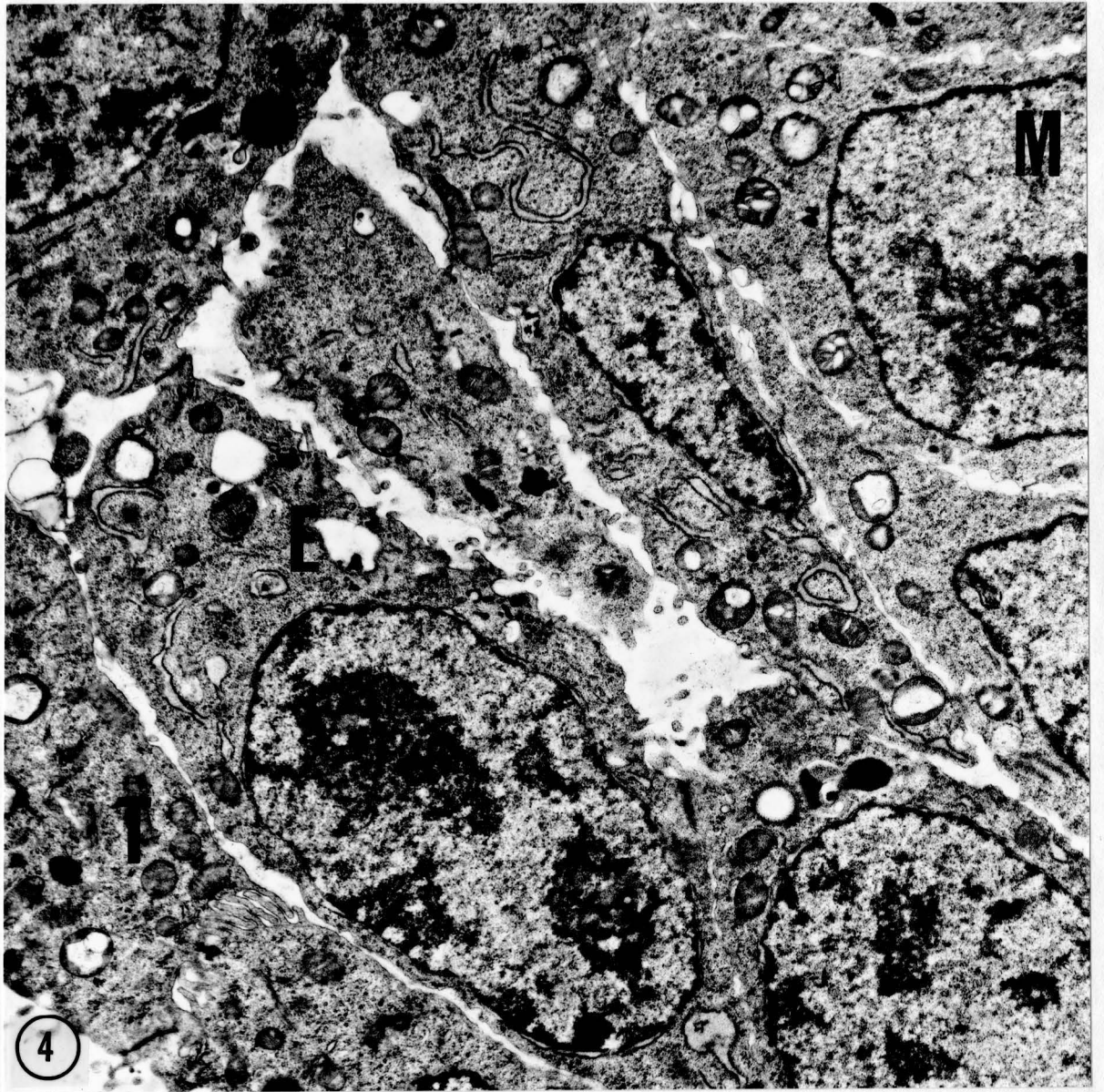


PLATE III

EXPLANATION OF FIGURE

Figure 4. A higher power electron micrograph of the same field of section observed in Figure 3 showing trophoblast (T), inner mass (M), and endoderm (E) cells. X12,000.



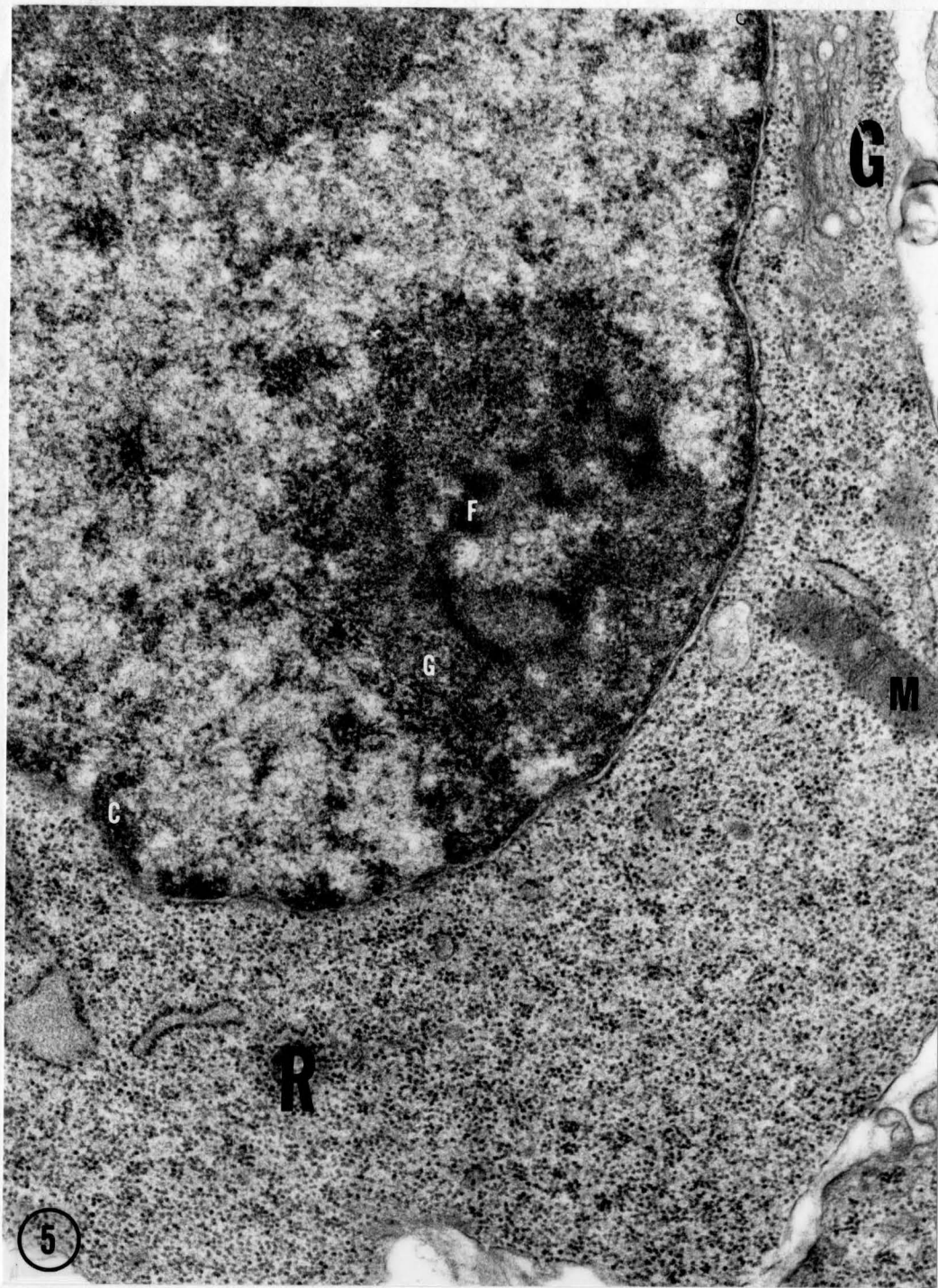


PLATE IV

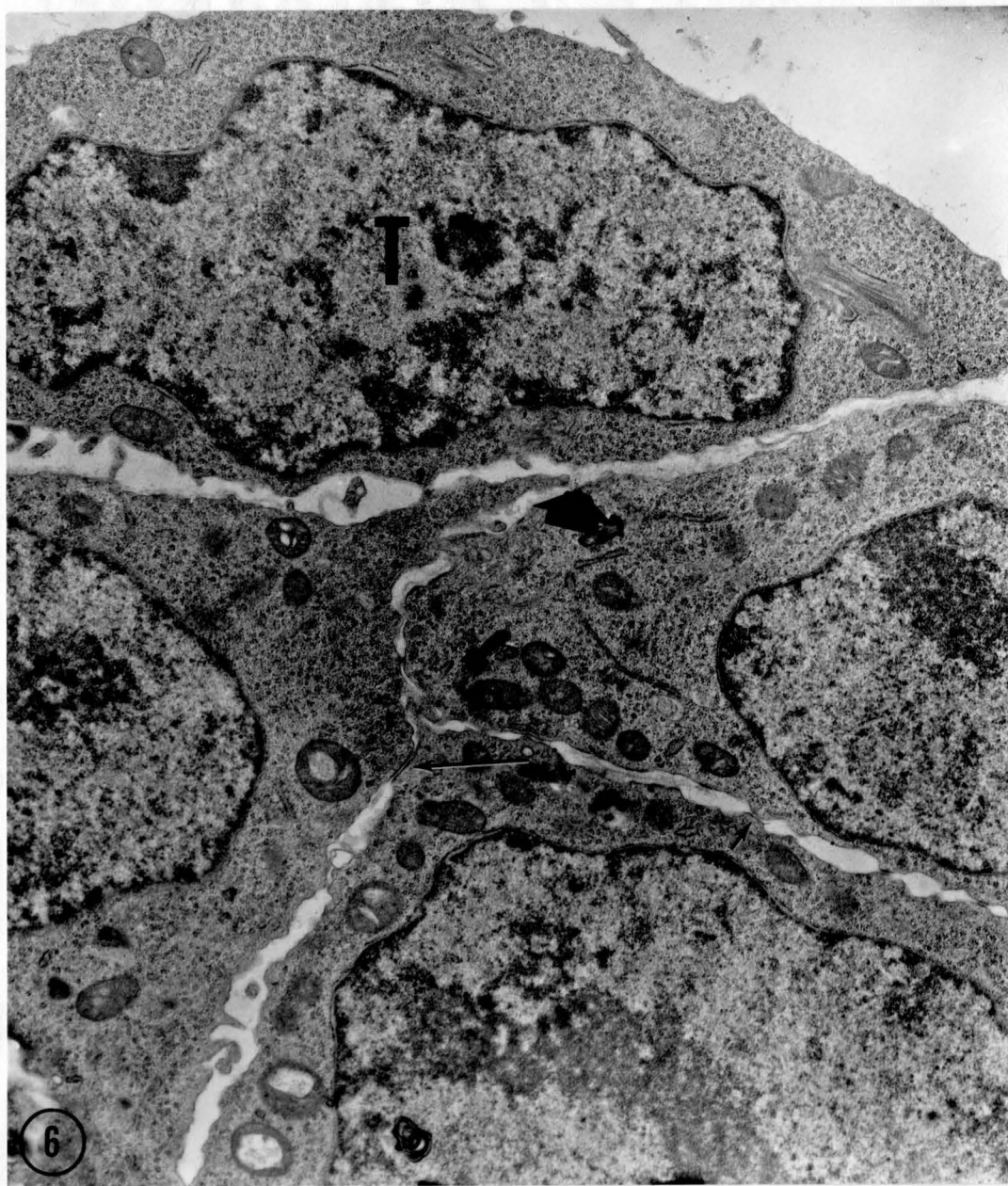
EXPLANATION OF FIGURE

Figure 5. Section of an inner mass or embryonic cell of an in vivo developed late preimplantation mouse blastocyst containing abundant ribosomes (R), some elements of rough endoplasmic reticulum, and a stack of 4 to 5 cisternae representing the Golgi complex (G). Note the fibrillar (F) and granular (white G) areas of the nucleolus, the condensed chromatin (C) lining the inner nuclear membrane, and the ribosomes attached to the outer nuclear membrane. M, mitochondrion. X30,000.

PLATE V

EXPLANATION OF FIGURE

Figure 6. Section through the embryonic pole of an in vivo grown late preimplantation mouse blastocyst. Note the loose apposition (heavy arrow) of the trophoblast (T) and adjacent inner mass cells. Zonulae adherentes (thin arrows) are responsible for maintaining cell adhesion. Both cell types appear to be heavily laden with ribonucleoprotein particles (ribosomes). X12,000.



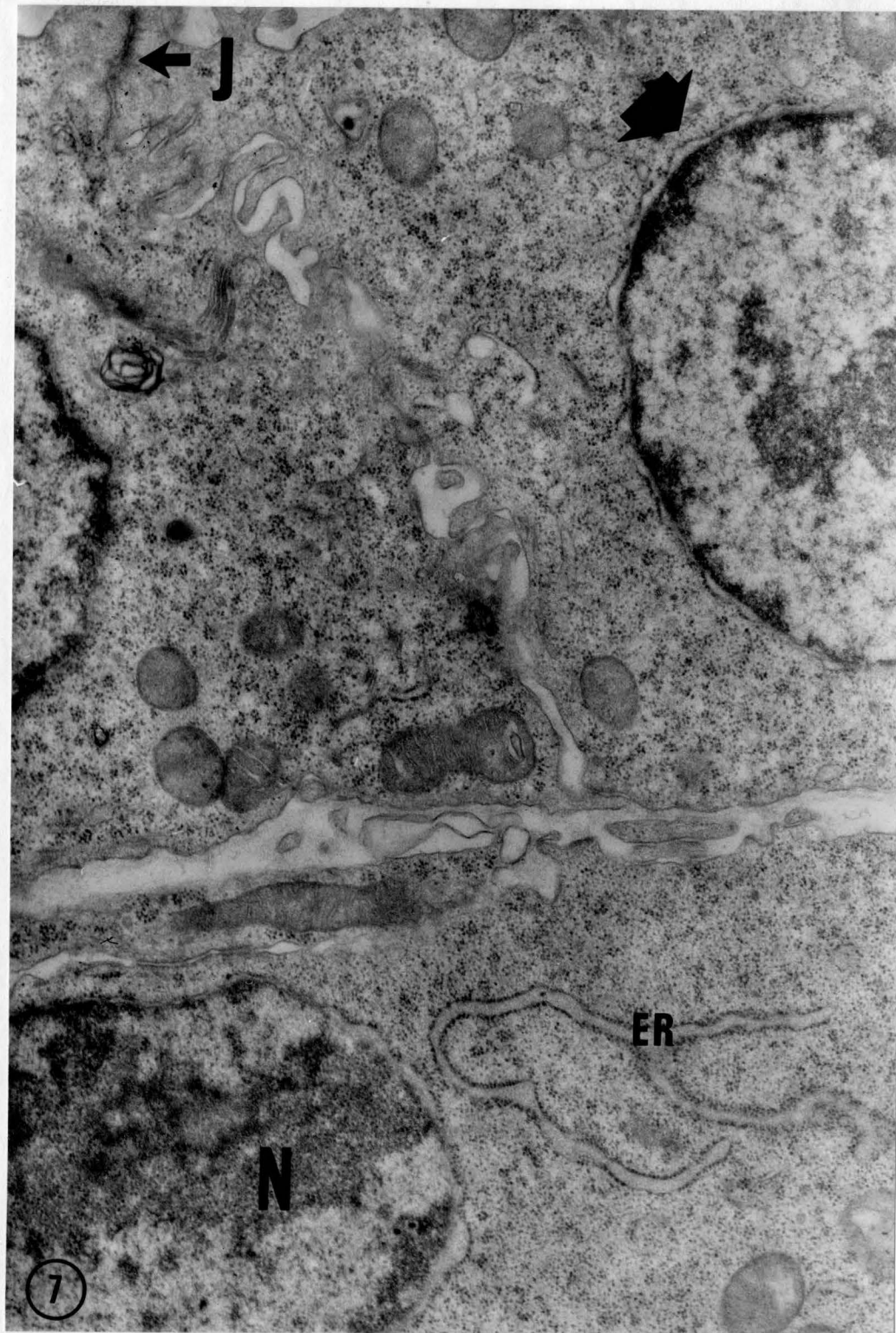


PLATE VI

EXPLANATION OF FIGURE

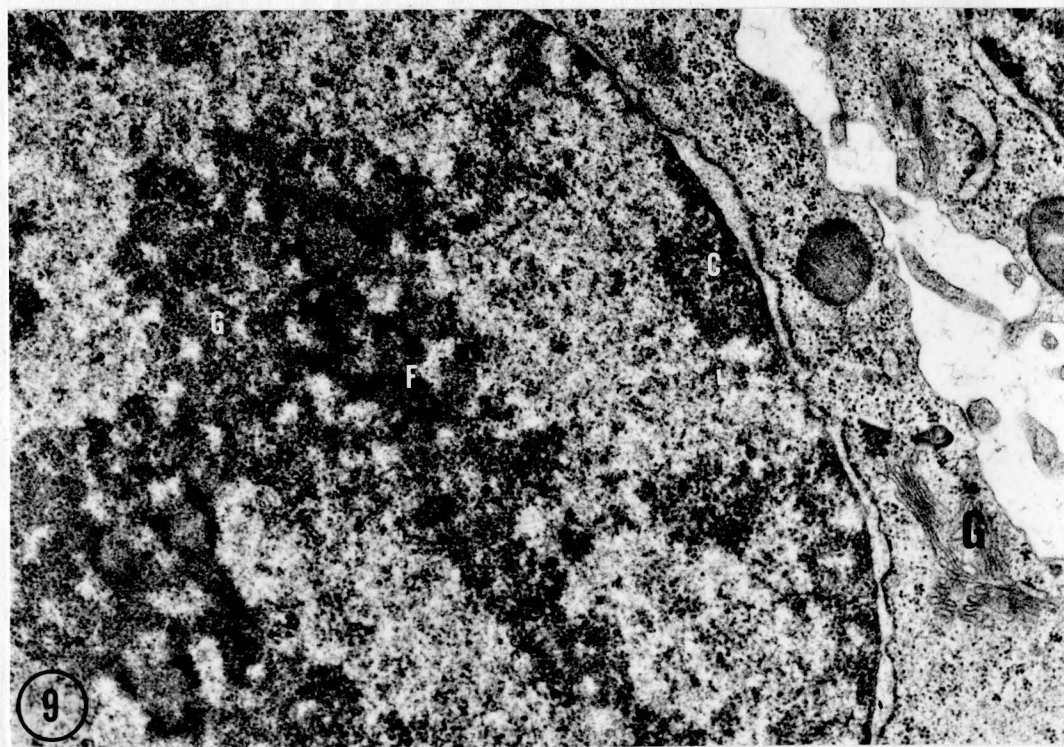
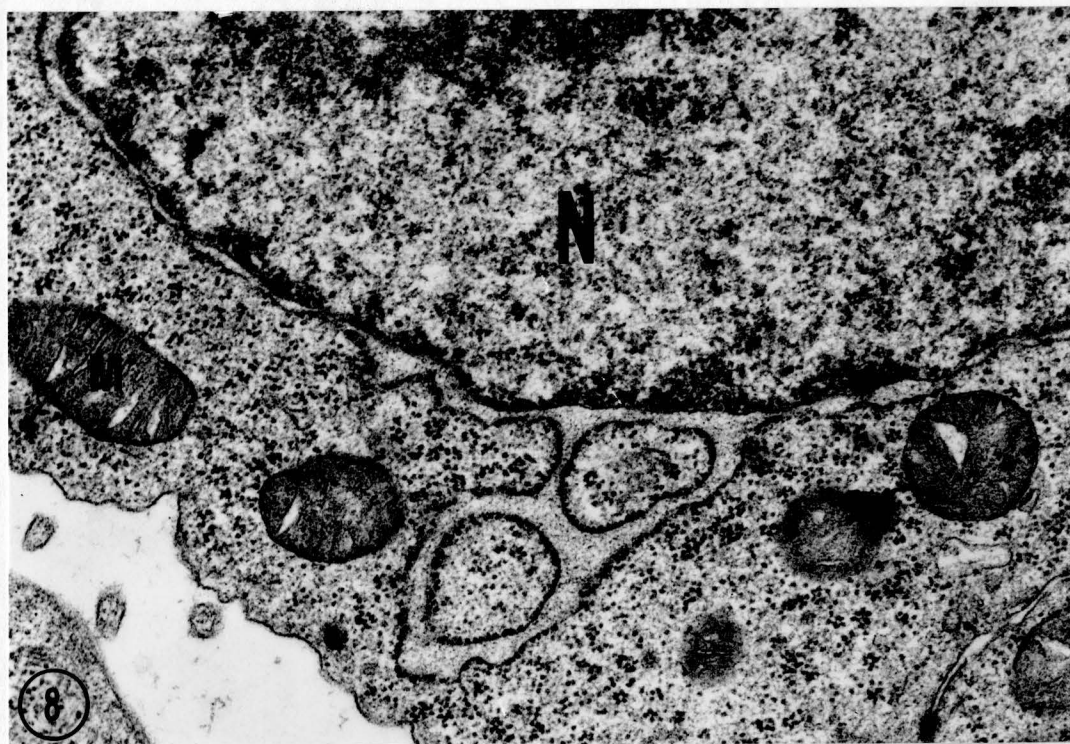
Figure 7. In vivo developed late preimplantation mouse blastocyst. This section contains a portion of a junctional complex (J) at the apical end of two trophoblast cell lateral borders. Microvilli are located immediately below the junctional complex. The heavy arrow indicates the distended perinuclear cisterna of one trophoblast cell. The trophoblast cell on the left contains microtubules and a myelin-like figure. The inner mass cell in the lower half of the electron micrograph has a well-differentiated nucleolus (N) and tubules of rough endoplasmic reticulum (ER) can also be observed. X22,000.

PLATE VII

EXPLANATION OF FIGURES

Figure 8. In vivo developed late preimplantation mouse blastocyst. The ribosome studded outer nuclear membrane of this inner mass cell is continuous with channels of rough endoplasmic reticulum. In addition, the perinuclear cisterna of the inner mass cell is distended at several locations. Two of the mitochondria (M) contain electron-dense inclusions which may be matrix granules. N, nucleus. X30,000.

Figure 9. In vivo developed late preimplantation mouse blastocyst. The nucleus of this inner cell mass cell contains fibrillar (F) and granular (white G) nucleolar portions, and condensed chromatin lines the inner nuclear membrane (C). Note that the outer nuclear membrane is heavily lined with ribosomes. The Golgi complex (black G) is not distended. X22,000.



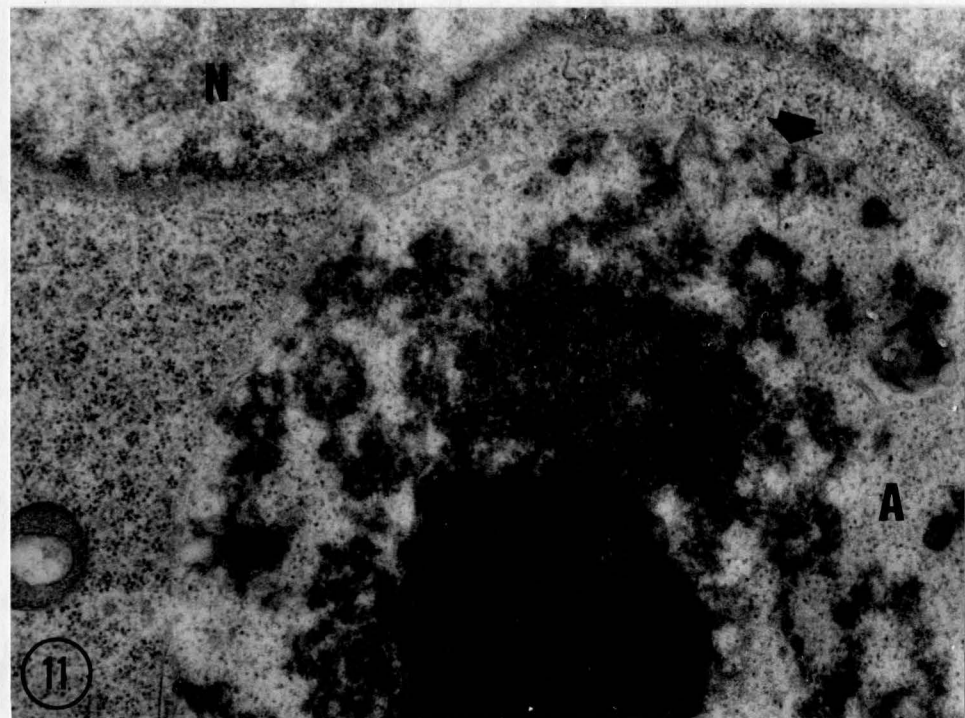
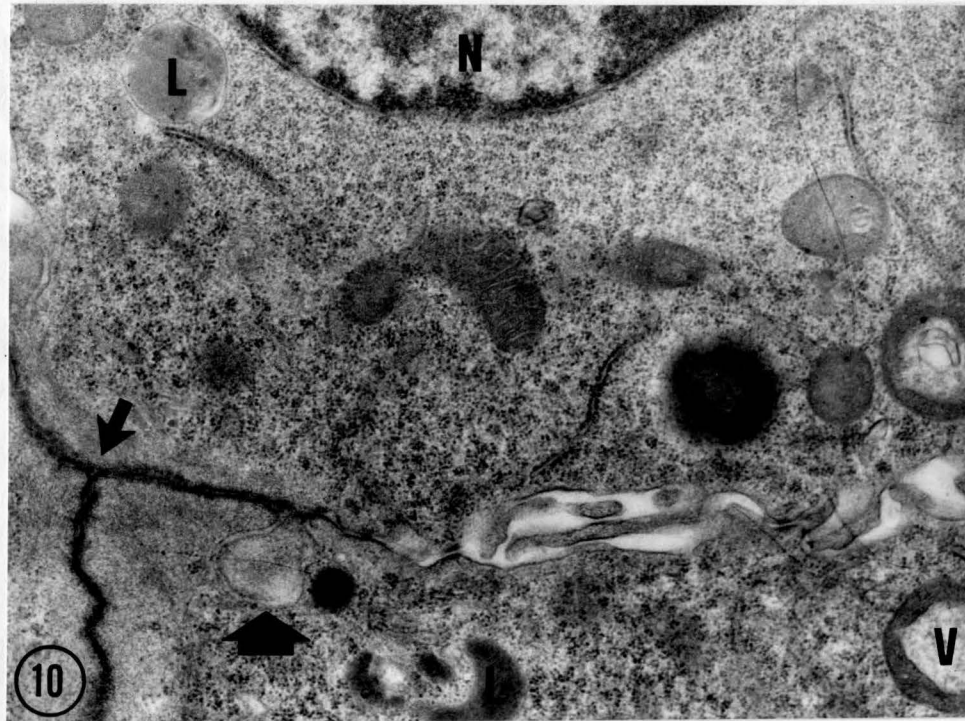


PLATE VIII

EXPLANATION OF FIGURES

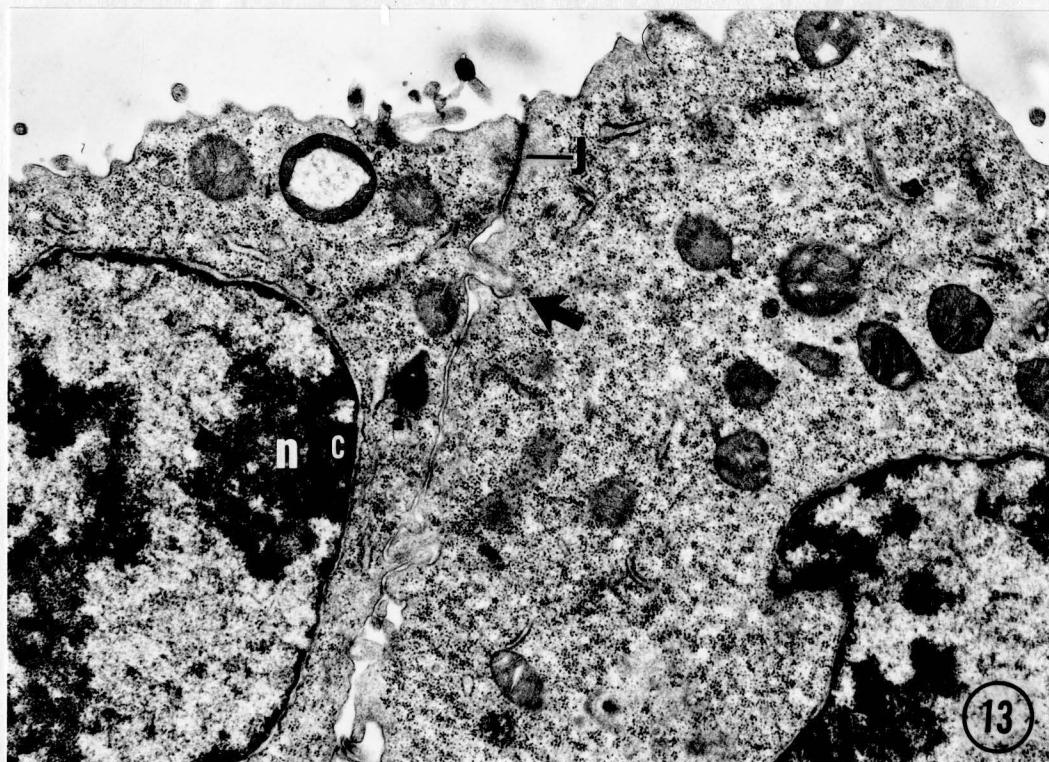
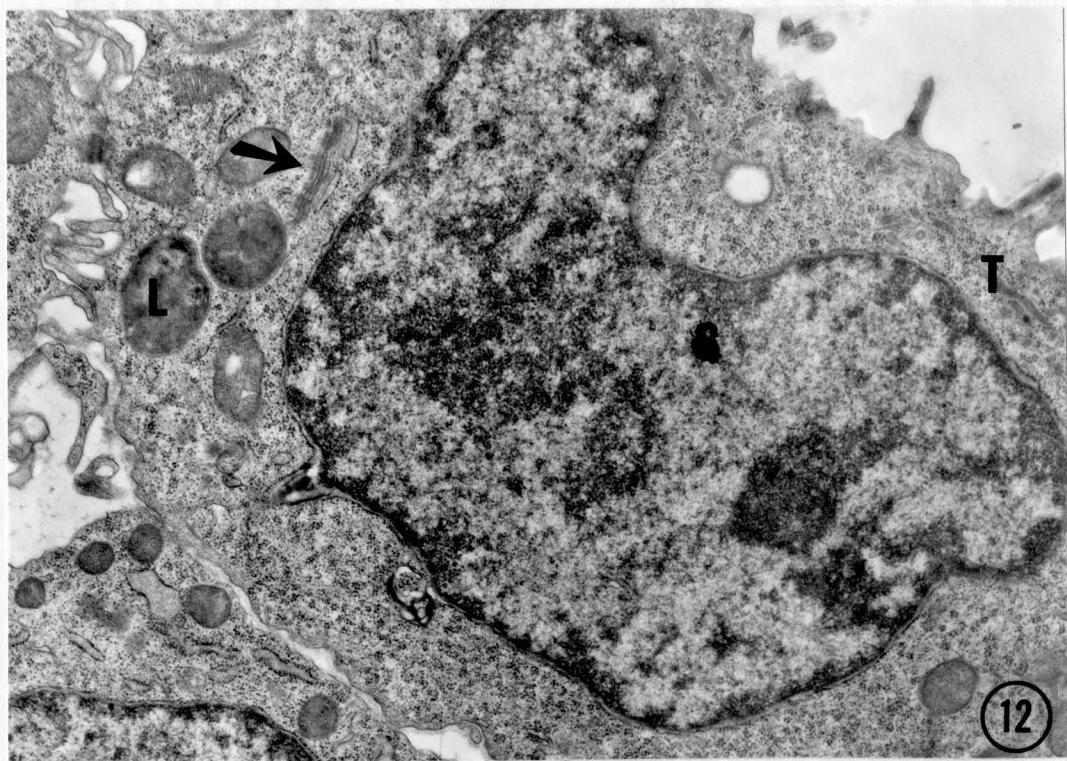
Figure 10. In vivo developed late preimplantation mouse blastocyst. This electron micrograph shows a portion of a junctional complex between the lateral cell borders of three trophoblast cells (small arrow). Note the tongue and groove appearance formed by a microvillus of one of the trophoblast cells (large arrow). N, nucleus; L, lysosome-like body; V, vacuolated mitochondrion. X22,000.

Figure 11. Autophagic vacuole (A) within a trophoblast cell of an in vivo developed late preimplantation mouse blastocyst. Note the limiting membrane (arrow) which separates the vacuole from the cell cytoplasm. N, nucleus. X22,000.

PLATE IX

EXPLANATION OF FIGURES

- Figure 12. In vivo developed late preimplantation mouse blastocyst. There is an intimate association between a stack of Golgi cisternae and rough endoplasmic reticulum (arrow) in this trophoblast cell. The membrane of the endoplasmic reticulum which faces the Golgi complex is devoid of ribosomes. The endoderm cell in the lower left corner of the electron micrograph contains wide channels of rough endoplasmic reticulum. C, condensed chromatin; T, microtubules; L, lysosome-like structure. X14,000.
- Figure 13. In vivo developed late preimplantation mouse blastocyst. A junctional complex (J) can be observed between two trophoblast cells. A microvillus of one trophoblast cell forms a "tongue and groove" connection with the other trophoblast cell (arrow). Both cells contain numerous ribosomes and some tubules of endoplasmic reticulum. The trophoblast cell at the left contains definitive nucleolus (n) and nucleolar associated chromatin (C), as well as a vacuolated mitochondrion. M, mitochondrion. X14,000.



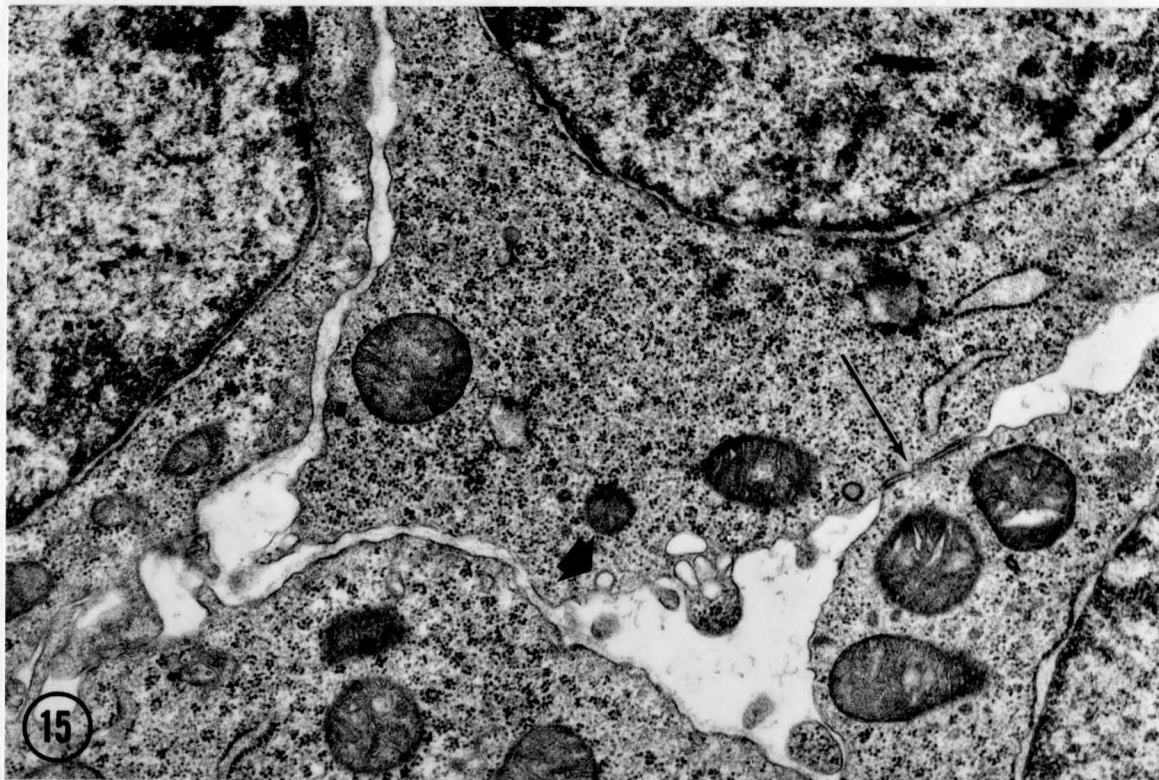
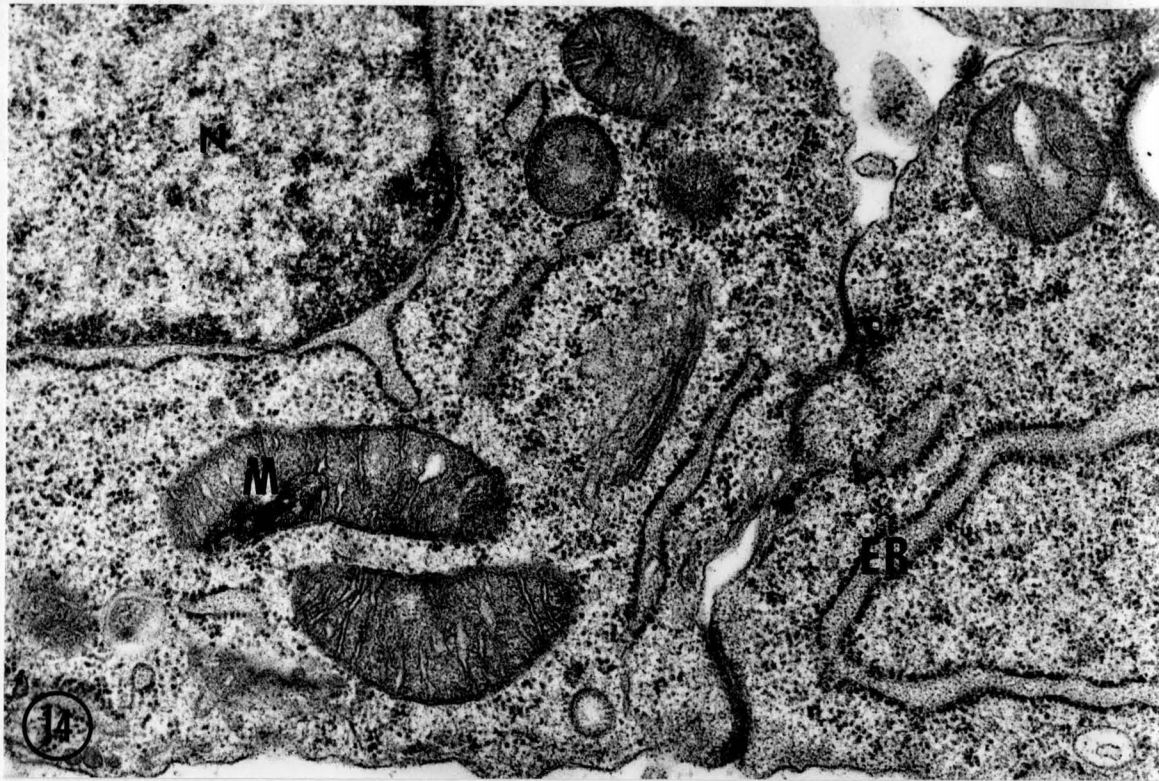


PLATE X

EXPLANATION OF FIGURES

Figure 14. Section through two trophoblast cells of an in vivo developed late preimplantation mouse blastocyst. In the cell on the left, note the connection of the perinuclear cisterna with the rough endoplasmic reticulum. Two mitochondria (M) are elongate and they contain a relatively large number of cristae. N, nucleus; ER, rough endoplasmic reticulum. X22,000.

Figure 15. Section through several inner mass cells of the in vivo developed late preimplantation mouse blastocyst. Large numbers of ribonucleoprotein particles are present in the cytoplasm of these cells, and they also line the outer nuclear membranes. Note the "tight" junction or zonula adherens (thin arrow). The mitochondria have an electron-dense matrix. Pinocytotic caveola (wide arrow). X22,000.

PLATE XI

EXPLANATION OF FIGURE

Figure 16. Junction between two trophoblast cells at the abembryonic pole of an in vivo developed late mouse blastocyst. All three components of a typical junctional complex (zonula occludens, zonula adherens, and macula adherens) are visible, as well as microvilli at the basal end of the lateral trophoblast cell borders (arrow). One lysosome-like body (L) appears to be fusing with an autophagic vacuole. V, vacuolated mitochondrion; N, nucleus, X11,000.



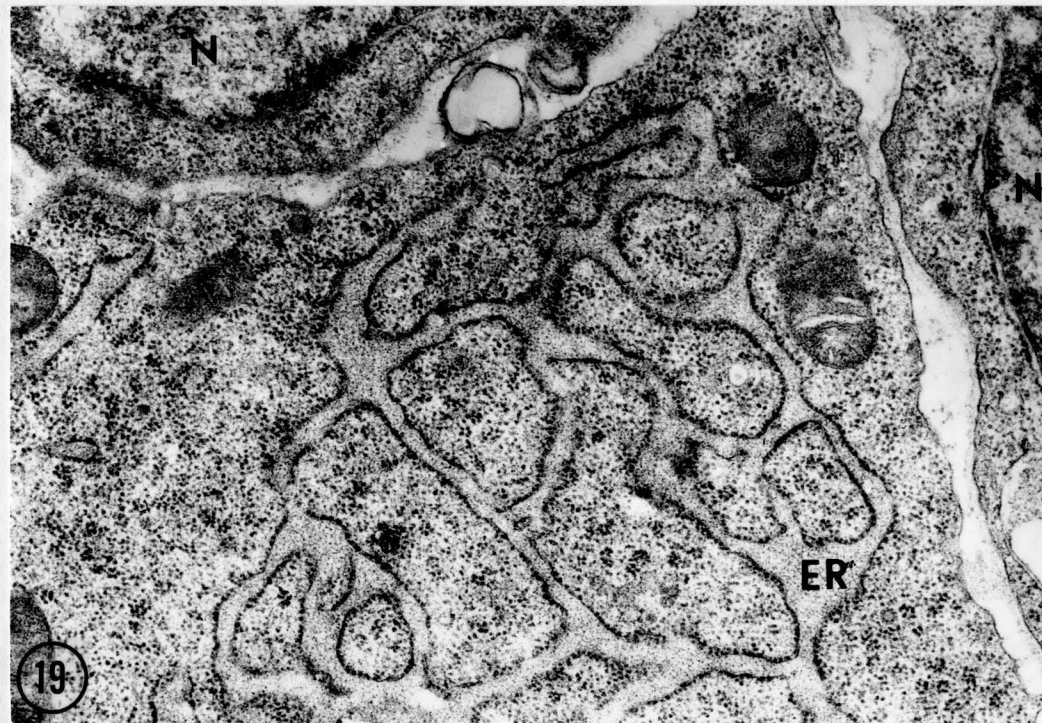
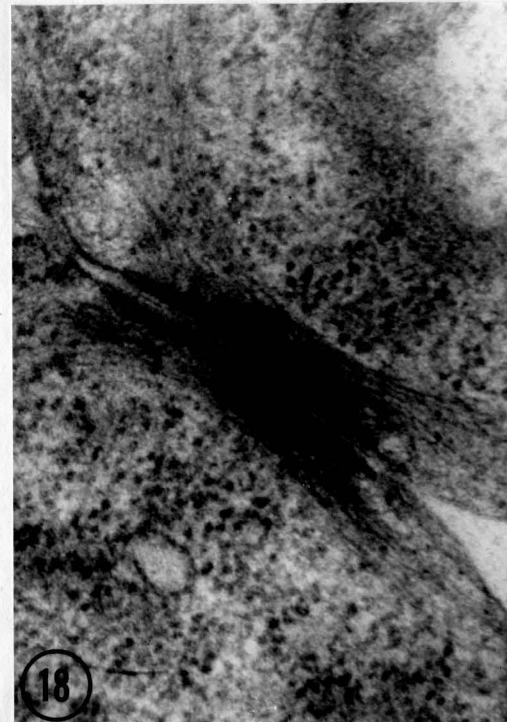


PLATE XII

EXPLANATION OF FIGURES

- Figure 17. In vivo developed late preimplantation mouse blastocyst. Small, double-membrane lined bodies (approximately 100 to 120 μ in diameter) appear to be formed and released from a lysosome-like body (L). M, mitochondrion. X48,000.
- Figure 18. Desmosome (macula adherens) in junctional complex between lateral cell borders of two trophoblast cells of an in vivo developed late preimplantation mouse blastocyst. Note the characteristic thickening of the inner leaflet of each membrane and the tonofilaments covering from the adjacent cytoplasm. X68,000.
- Figure 19. In vivo developed late preimplantation mouse blastocyst. Anastomosing and interconnecting channels of rough endoplasmic reticulum occupy a large amount of the cytoplasm of this endoderm cell. ER, endoplasmic reticulum; N, nuclei of adjacent inner mass cells. X14,000.

PLATE XIII

EXPLANATION OF FIGURE

Figure 20. Electron micrograph of endoderm (upper) and inner mass cells (lower) of an in vivo developed late preimplantation mouse blastocyst. Note the wide cisternae of rough endoplasmic reticulum in the endoderm cell (ER), as well as the myelin-like figures in the vicinity of the nucleus (thick arrow). All cells are heavily laden with ribonucleoprotein particles. Caveolae, indicative of micropinocytosis, are also observed in this section (small arrow). Note also the large intercellular spaces. N, nucleus; G, Golgi complex; M, mitochondrion; L, lysosome-like body. X14,000.

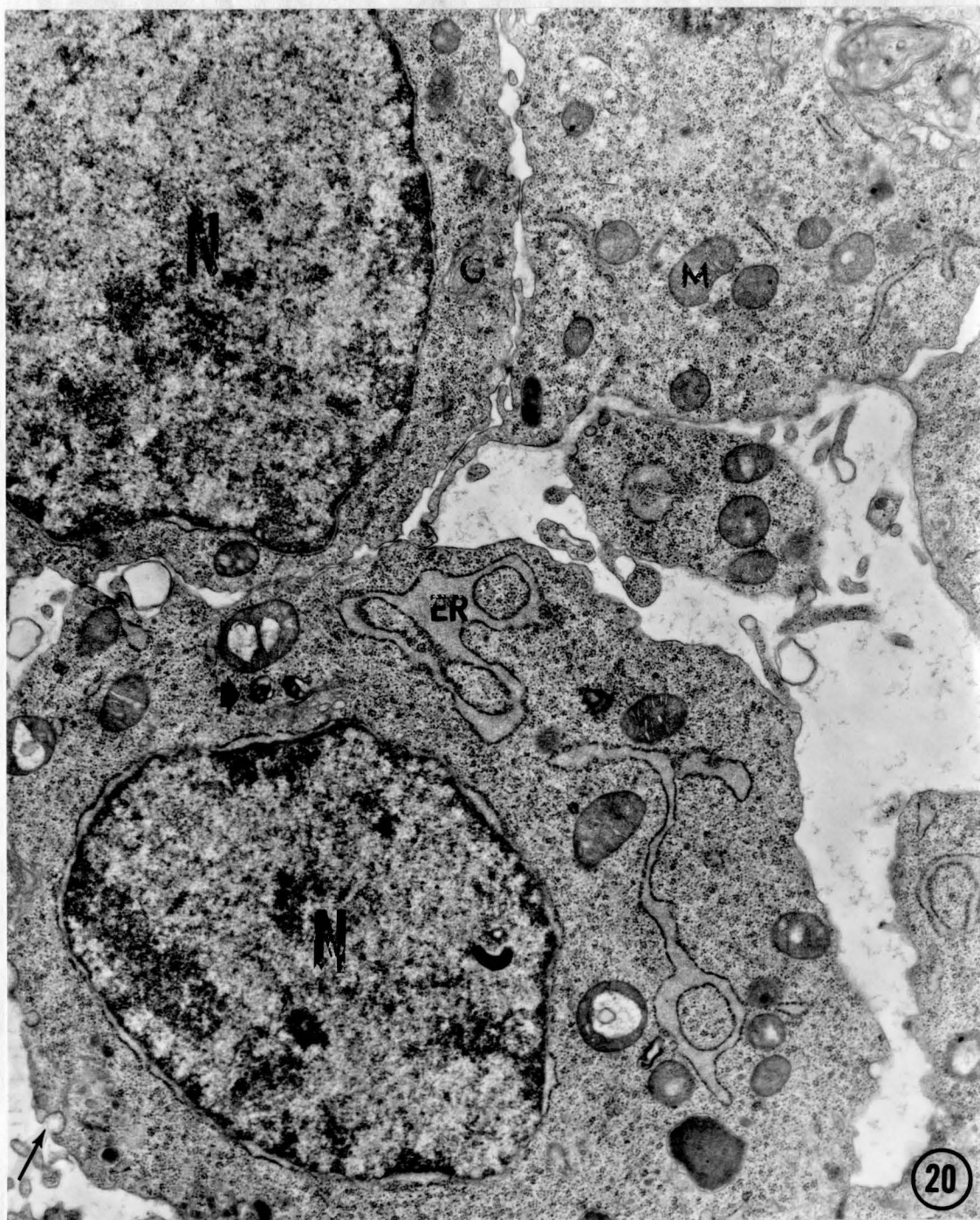


PLATE XIV

EXPLANATION OF THE FIGURES

Figure 21. Electron microscope autoradiogram of an inner mass cell of an in vivo developed late preimplantation mouse blastocyst which was exposed to tritiated leucine for one hour in vitro. In addition to the cytoplasm, developed grains appear mainly over the non-condensed chromatin or euchromatin (E) of the nucleoplasm and over the nucleolus (N). H, heterochromatin. X14,000.

Figure 22. Electron microscope autoradiogram of a trophoblast cell of an in vivo developed late preimplantation mouse blastocyst which was exposed to tritiated leucine for one hour in vitro. One developed silver grain appears over the granular portion of the nucleolus (N). H, heterochromatin. X 22,000.

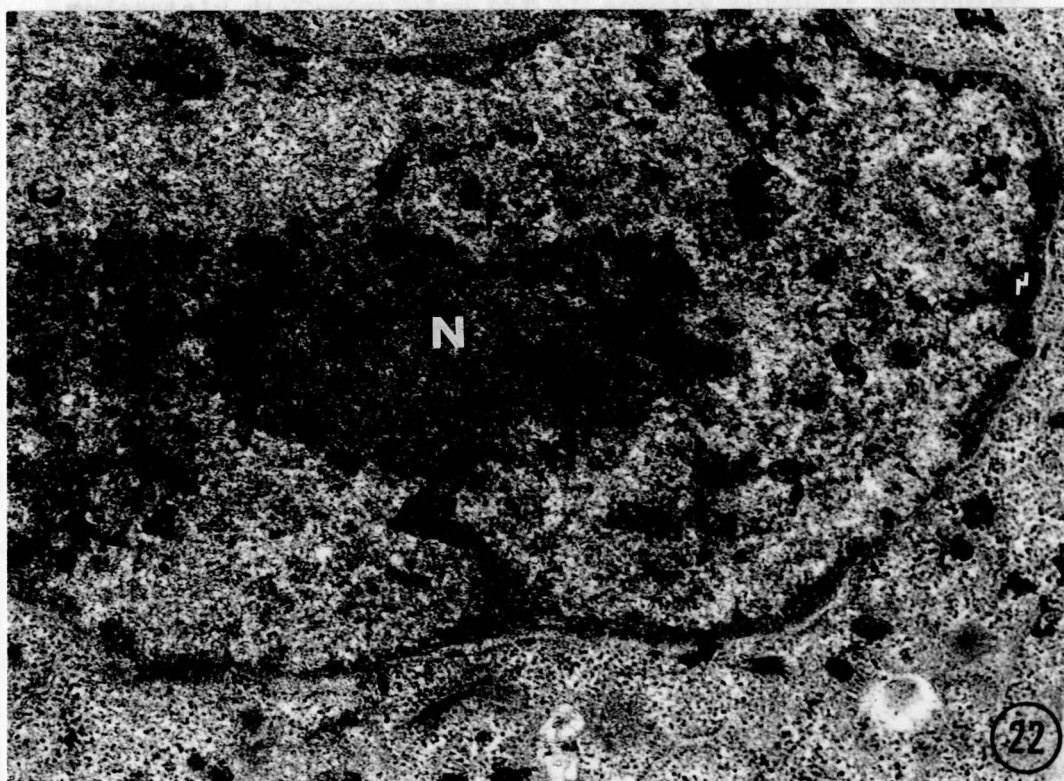
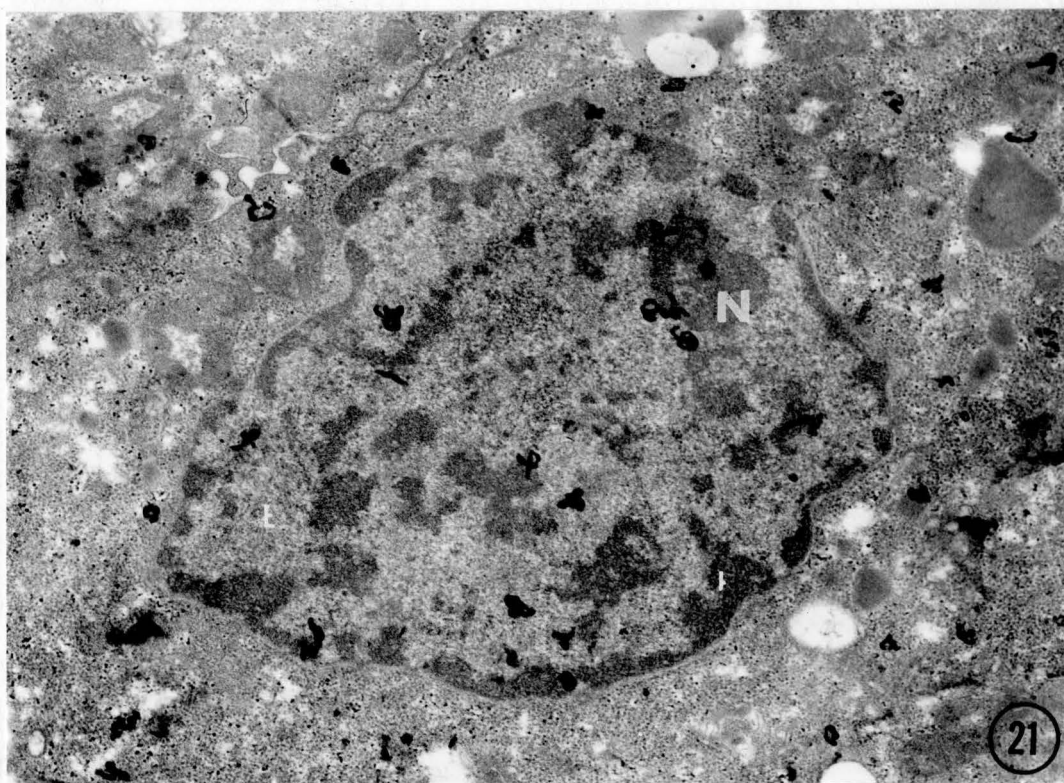
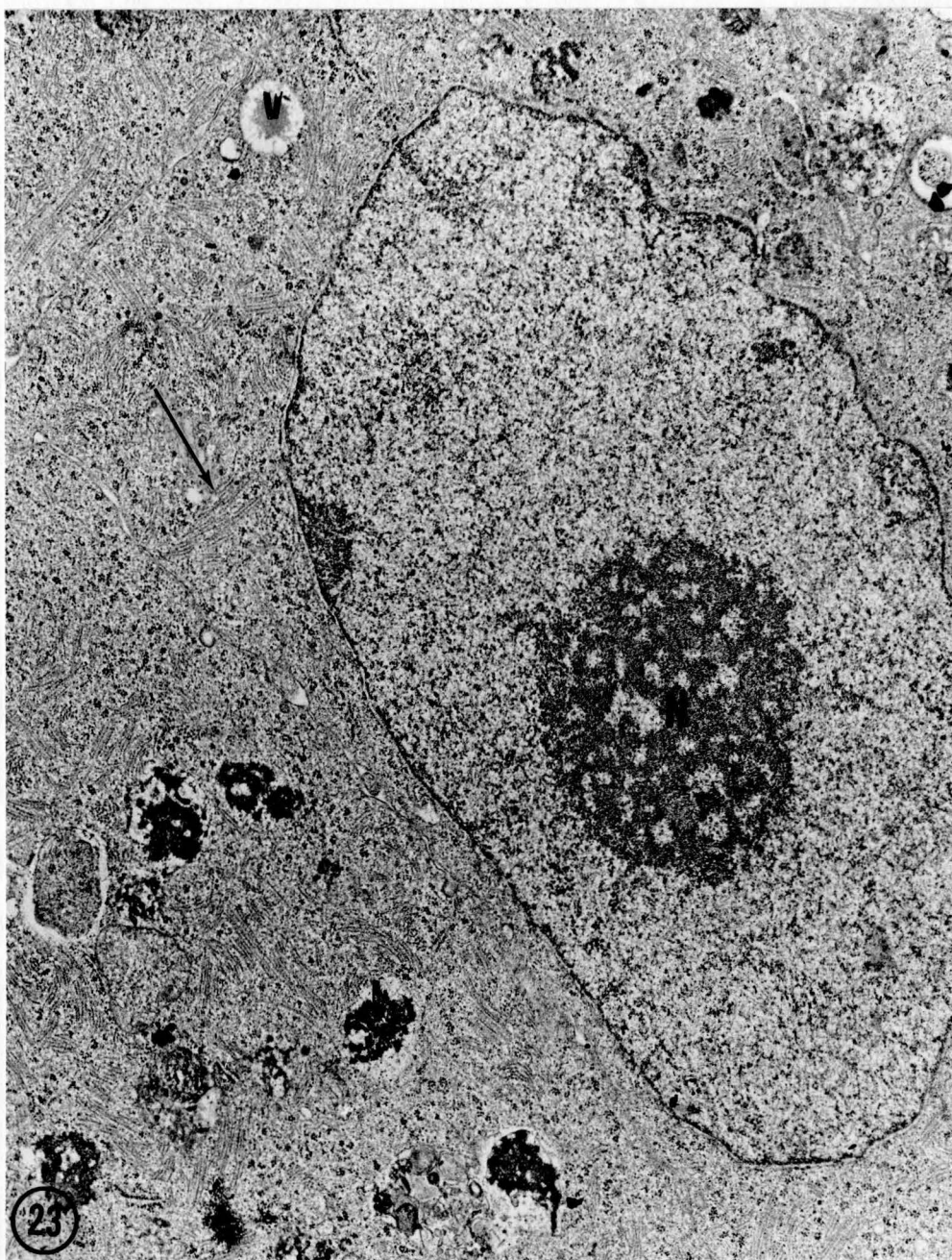


PLATE XV

EXPLANATION OF FIGURE

Figure 23. Trophoblast cell of a blastocyst grown from the two-cell stage after 72 hours cultivation in vitro. The nucleolus (N) is moderately differentiated but it appears somewhat reticular. Little heterochromatin or condensed chromatin lines the inner nuclear membrane or is found in the nucleoplasm. The perinuclear cisterna is not distended and few ribosomes line the outer nuclear membrane. The cytoplasm contains ribosomes which are clustered in small groups between fibrous strands (arrow). There appear to be fewer ribosomes in this cell than are found in cells of the in vivo developed blastocyst. The flocculent, electron-dense inclusions in the lower left corner of this electron micrograph are lysosome-like structures. V, cytoplasmic vesicles. X14,000.



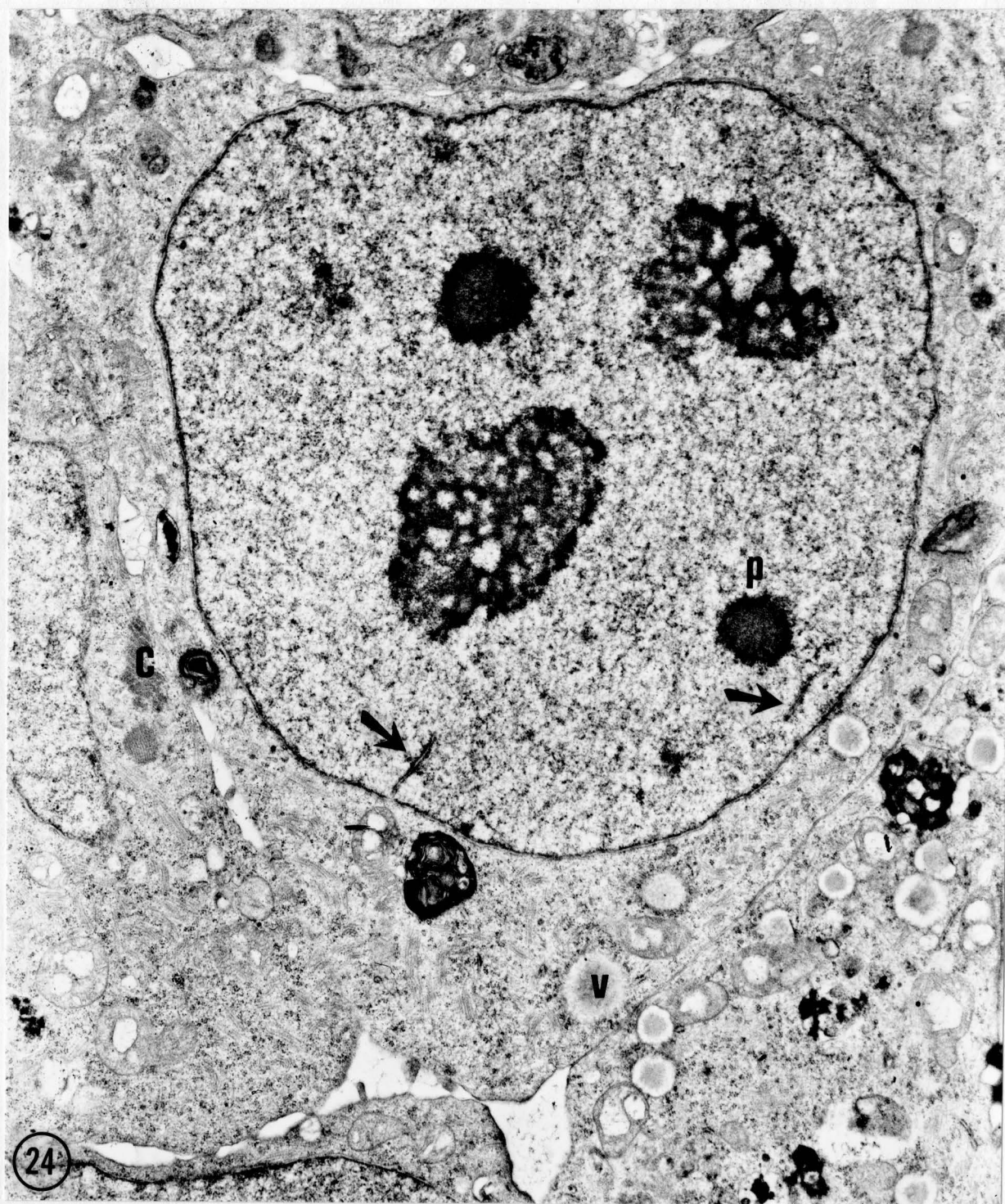


PLATE XVI

EXPLANATION OF FIGURE

- Figure 24. Inner mass cell of a blastocyst cultured 72 hours in vitro. Nuclear morphology appears less differentiated than that observed in the in vivo developed late preimplantation blastocyst. Two primary, undifferentiated nucleoli (P) and two reticulated, moderately differentiated nucleoli consisting of fibrillar and granular areas are observed in the nucleoplasm. Intranuclear annulate lamellae, not found in cells of the in vivo developed late preimplantation blastocyst, are seen perpendicular and parallel to the nuclear envelope (arrows). Note the fibrous strands interspersed between small groups of ribosomes. Little heterochromatin lines the inner nuclear membrane or is found in foci in the nucleoplasm. V, cytoplasmic vesicle; C, crystalloid. X14,000.

PLATE XVII

EXPLANATION OF FIGURES

- Figure 25. Blastocyst formed from two-cell stage embryo which was cultured 72 hours in vitro. This inner mass cell contains an intranuclear annulate lamellar structure (IAL). X30,000.
- Figure 26. Myelin-like figure in a trophoblast cell of an embryo grown 72 hours in vitro from the late two-cell stage. Notice the clusters of ribosomes which are interspersed between the fibrous strands. Also note the desmosome (macula adherens) located in the upper left corner of the electron micrograph. X30,000.
- Figure 27. Inner mass cell of a blastocyst developed from the two-cell stage after culture for 72 hours in vitro. The nucleus contains a moderately differentiated nucleolus which is reticular in appearance. Intranuclear annulate lamellae (arrows) are associated with the nucleolus. C, Crystalloid. X30,000.

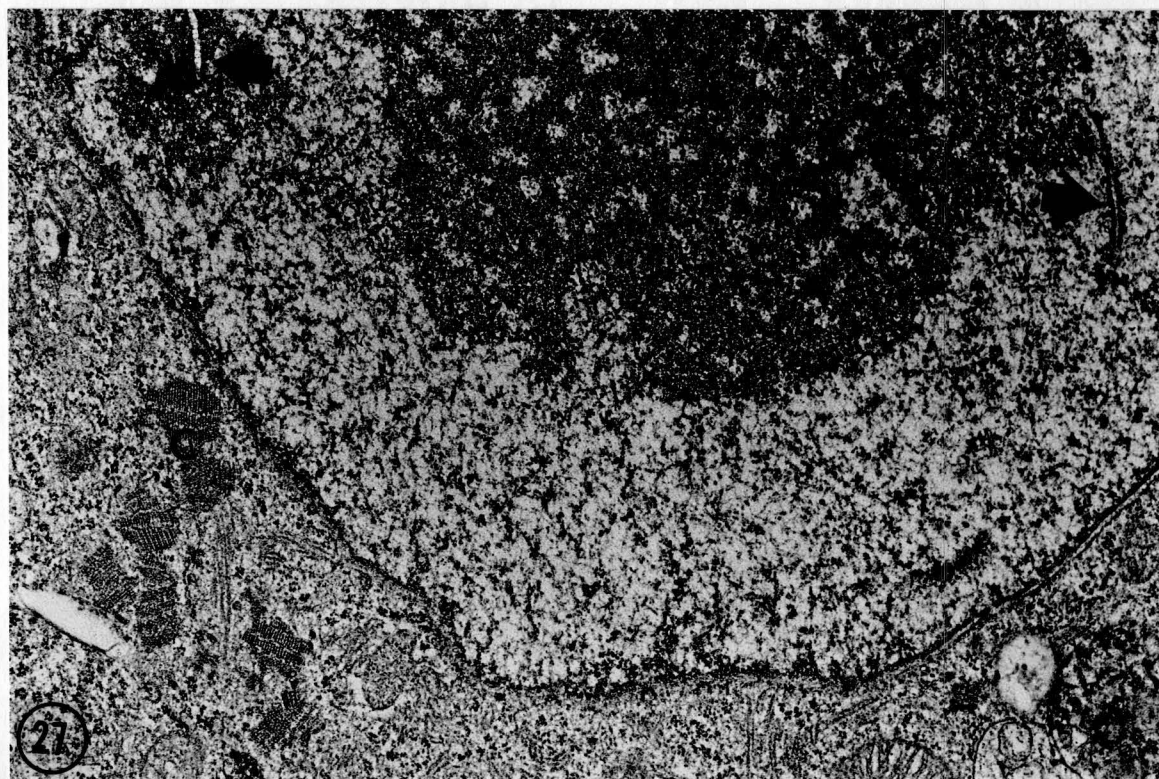
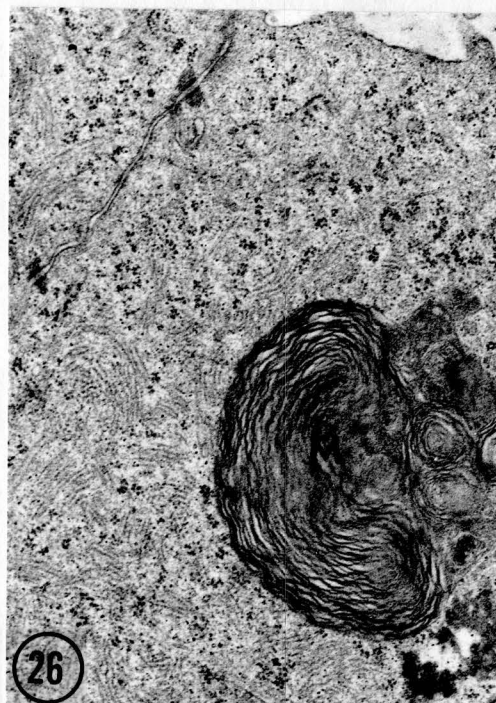


PLATE XVIII

EXPLANATION OF FIGURES

Figure 28. Electron micrograph of two trophoblast cells of an embryo grown 72 hours in vitro from the late two-cell stage. Note the groups of ribosomes which are interspersed between the numerous fibrous strands (F). D, desmosome; C, crystalloid. X30,000.

Figure 29. Trophoblast cell of a blastocyst grown 72 hours in vitro from the late two-cell stage. Ribosomes are found in small groups interspersed between the fibrous strands (F). Note the crystalloid inclusions which are somewhat surrounded by elements of rough endoplasmic reticulum. C, crystalloid. X30,000.

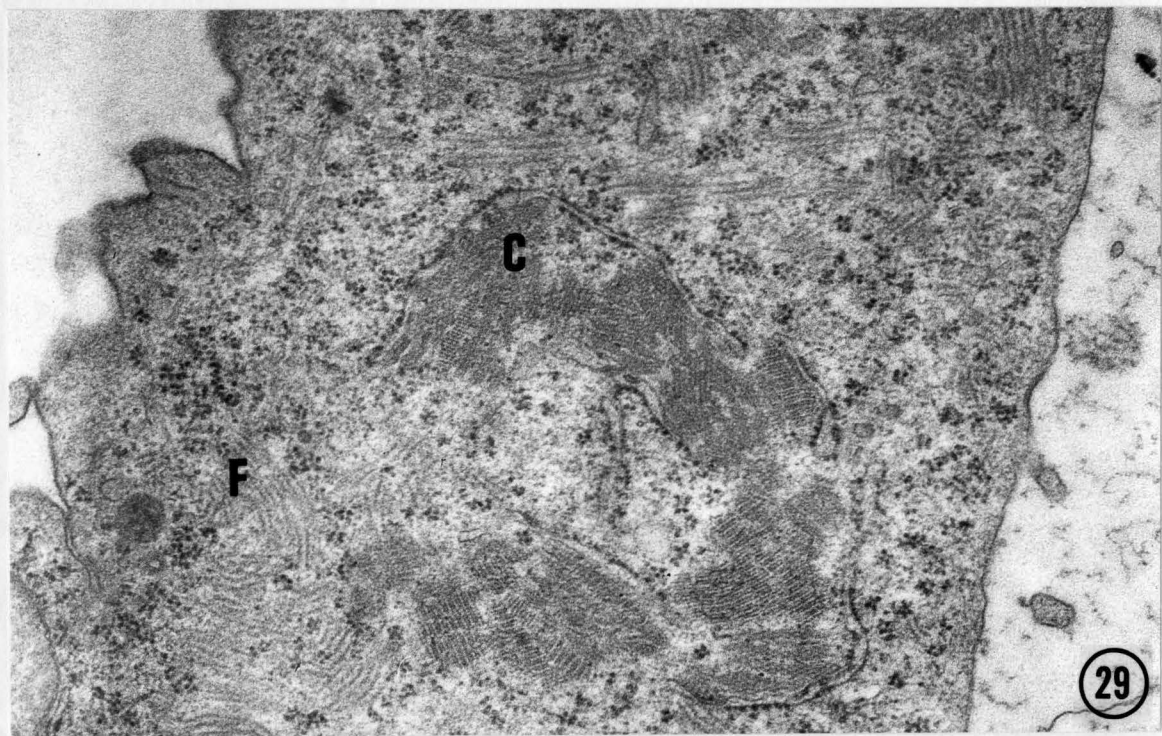
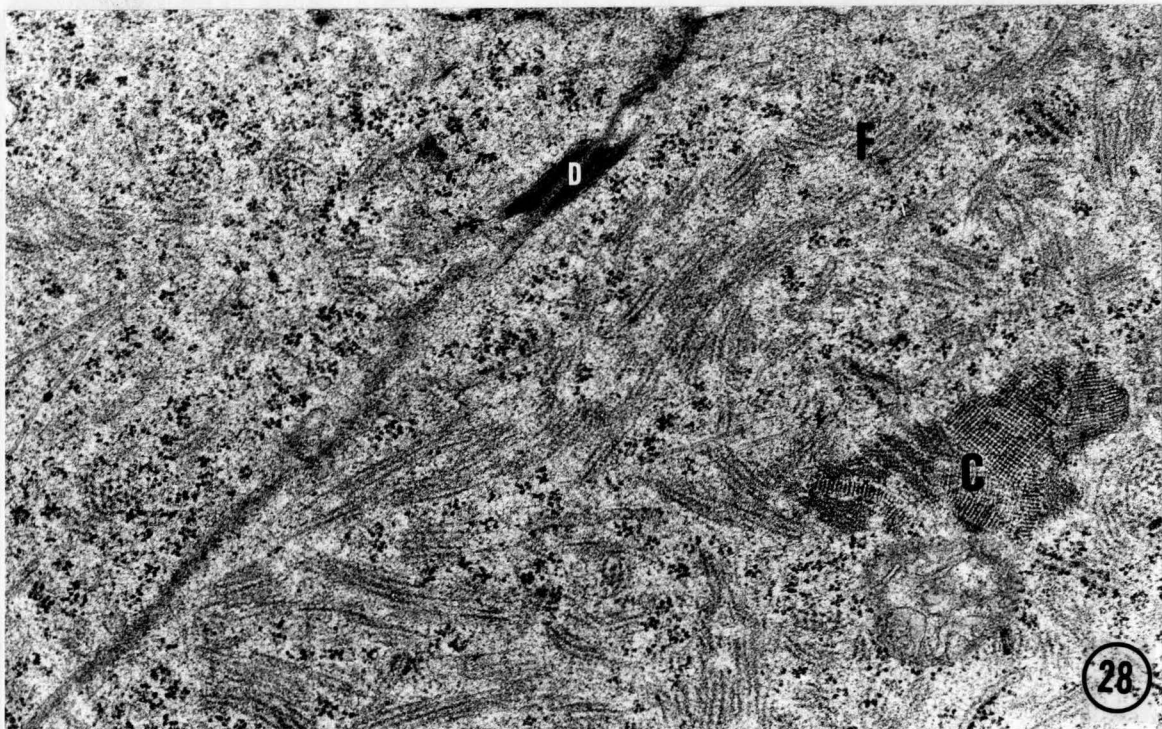


PLATE XIX

EXPLANATION OF FIGURES

- Figure 30. Trophoblast cell of a blastocyst cultured 72 hours in vitro. Notice the swollen mitochondria (M) containing several intracristal vacuoles. The mitochondrion in the upper left corner of the electron micrograph contains 10 to 12 parallel cristae while the other mitochondria are swollen or contain relatively few cristae. The Golgi complex is moderately enlarged and distended. X22,000.
- Figure 31. A strand of endoplasmic reticulum interposed between two mitochondria in a trophoblast cell of a blastocyst cultured 72 hours in vitro. Note the lack of ribosomes on the membranes of endoplasmic reticulum where they are intimately associated with mitochondria (M). The mitochondrion on the left has relatively few cristae. Also, observed the intimate association of the rough endoplasmic reticulum with a crystalloid inclusion (C). X68,000.
- Figure 32. Trophoblast cell of a blastocyst grown 72 hours in vitro. This figure reveals another close relationship between the rough endoplasmic reticulum and a mitochondrion (M). However, in this case the membrane of the reticulum adjacent to the mitochondrion retains some ribosomes. L, lysosome-like structure. X68,000.

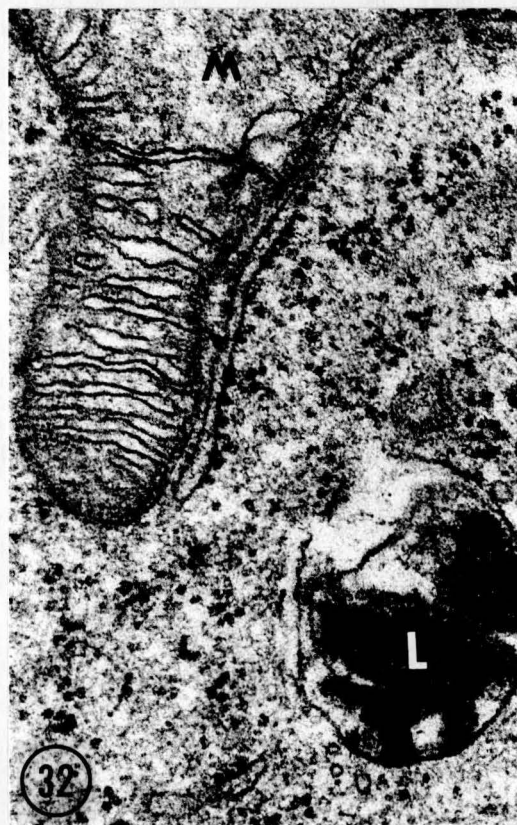
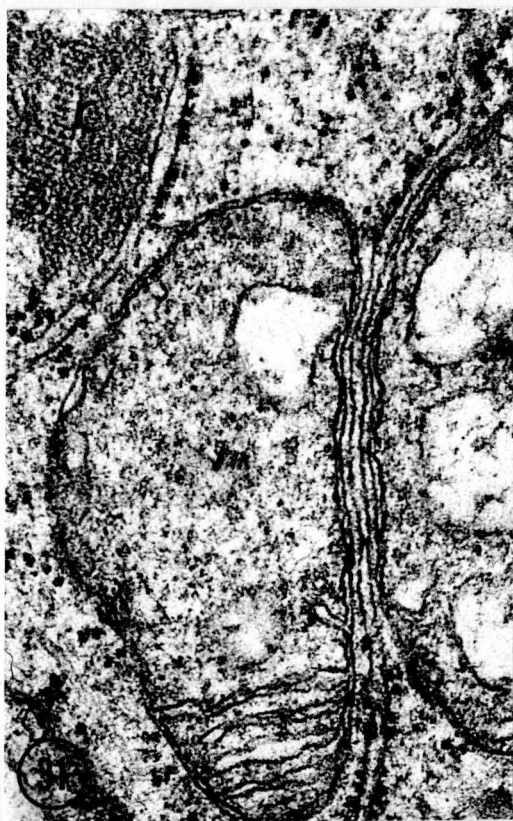
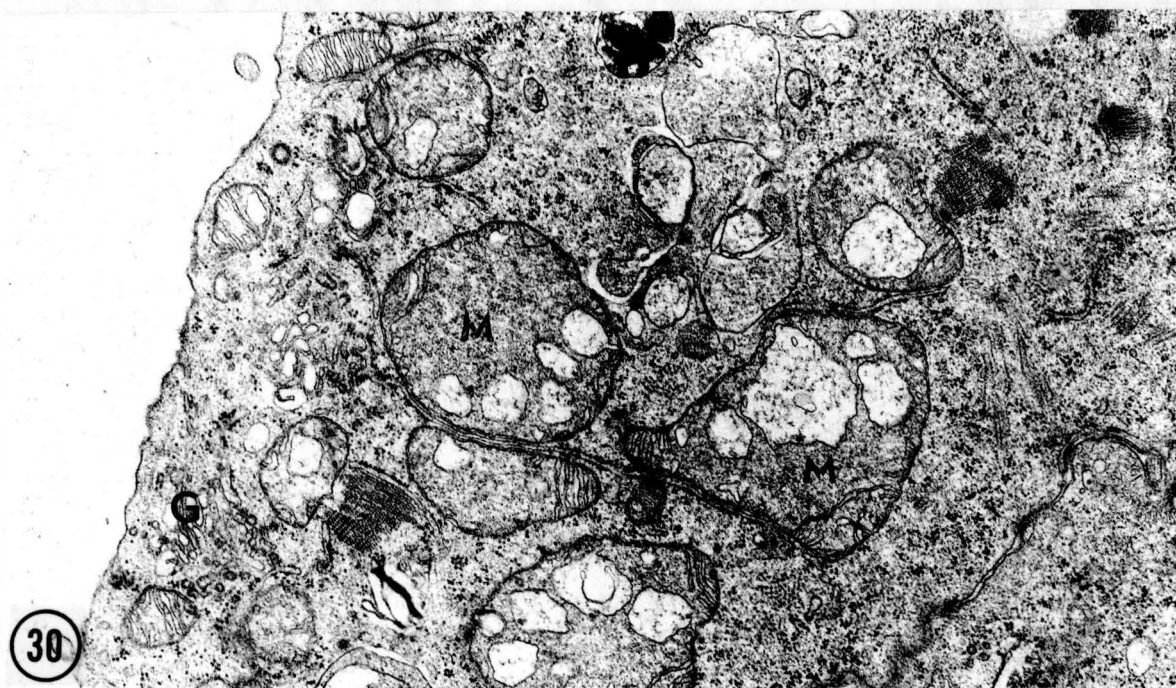


PLATE XX

EXPLANATION OF FIGURES

Figure 33. Blastocyst developed 72 hours in vitro from the late two-cell stage. This trophoblast cell contains pinocytotic vacuoles on its basal or blastocelic surface. These vacuoles appear to form by fusion of long, attenuated microvilli (arrows). N, nucleus. X30,000.

Figure 34. Junctions of trophoblast cells of blastocyst grown 72 hours in vitro from the late two-cell stage. Microvilli, usually present on lateral trophoblast cell borders in the in vivo developed late preimplantation blastocyst, are not present. V, cytoplasmic vesicles; L, lysosome-like body. X22,000.

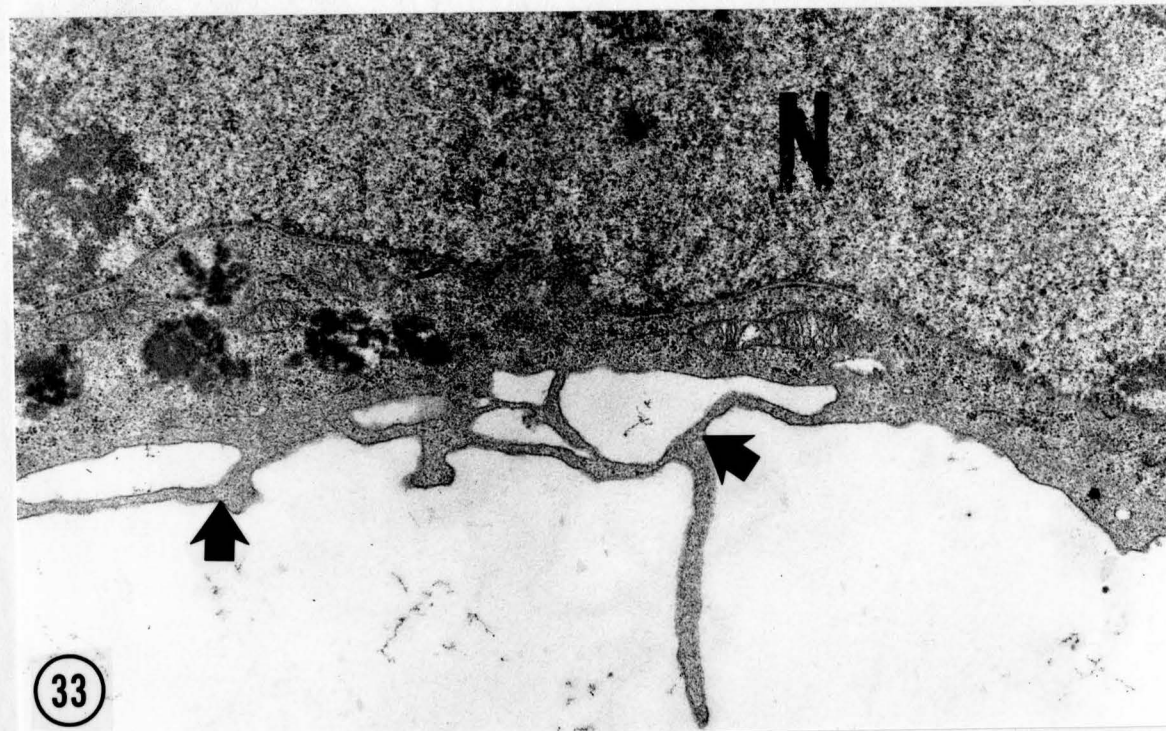


PLATE XXI

EXPLANATION OF FIGURE

Figure 35. Inner mass cells of a blastocyst developed 72 hours in vitro from the late two-cell stage. Notice the fibrous strands (arrow), the clusters of ribosomes, and the short elements of rough endoplasmic reticulum. L, lysosome-like structure; V, cytoplasmic vesicle; M, mitochondrion. X30,000.

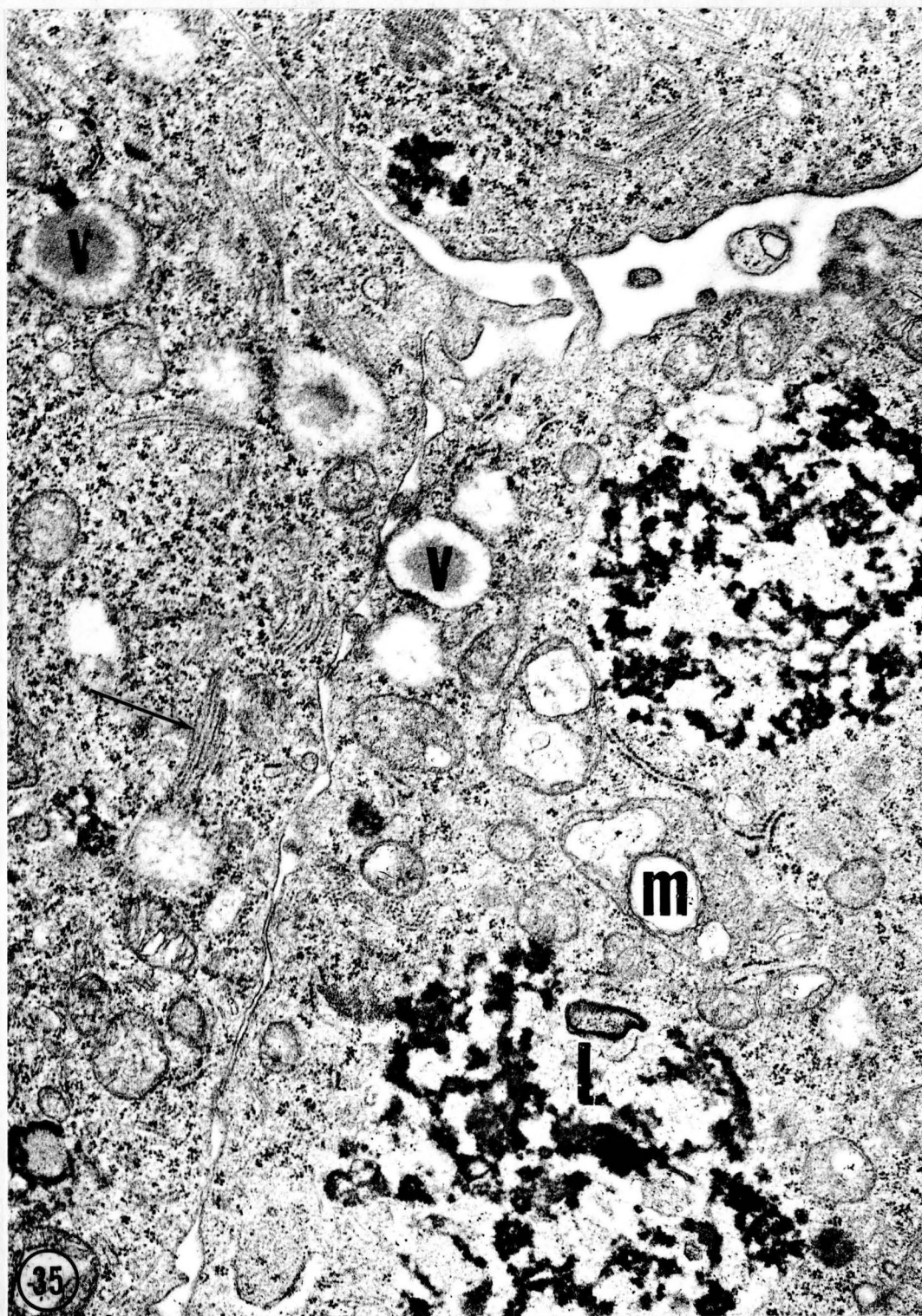


PLATE XXII

EXPLANATION OF FIGURE

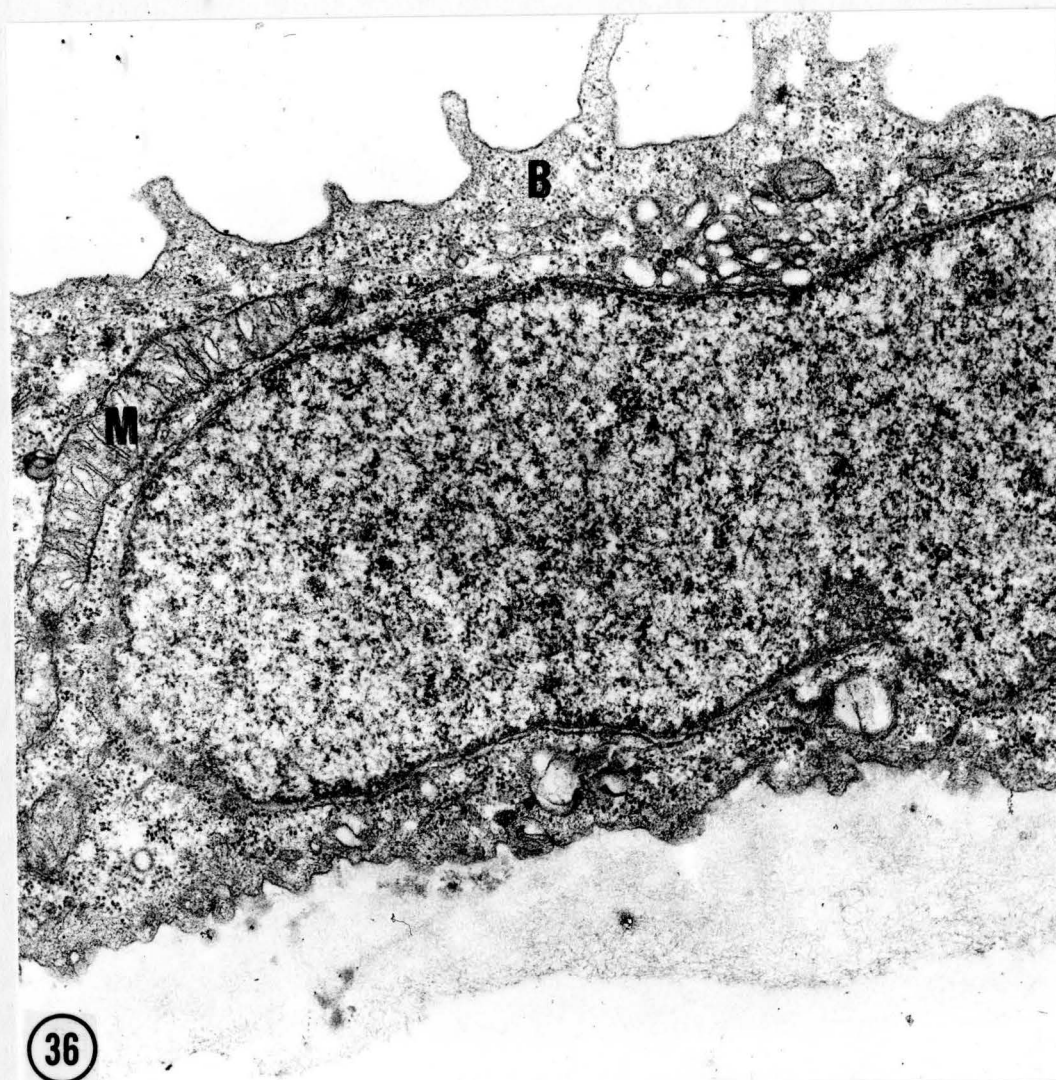
Figure 36. Trophoblast cell of blastocyst developed 92 hours *in vitro*. Microvilli line the basal or blastocelic surface (B). Slightly more heterochromatin lines the inner nuclear membrane than in 72-hour cultured embryos. Note the elongate mitochondrion (M). X30,000.

PLATE XXIII

EXPLANATION OF FIGURES

Figure 37. Trophoblast cell of blastocyst grown 92 hours in vitro from the late two-cell stage. Ribosomes are more abundant than in cells of 72-hour cultured blastocysts. Notice the ribosomes lining the outer nuclear membrane. One mitochondrion is extremely swollen (M) and it contains four intracristal vacuoles. Z, zona pellucida. Note the undulating cell membranes. X30,000.

Figure 38. Trophoblast cell of a blastocyst developed 92 hours in vitro from the late two-cell stage. Solid, electron-dense lysosome-like bodies, similar to those seen in the in vivo developed late preimplantation blastocyst, are present. Notice the undulating appearance of the cell membrane. M, mitochondrion; N, nucleus. X30,000.



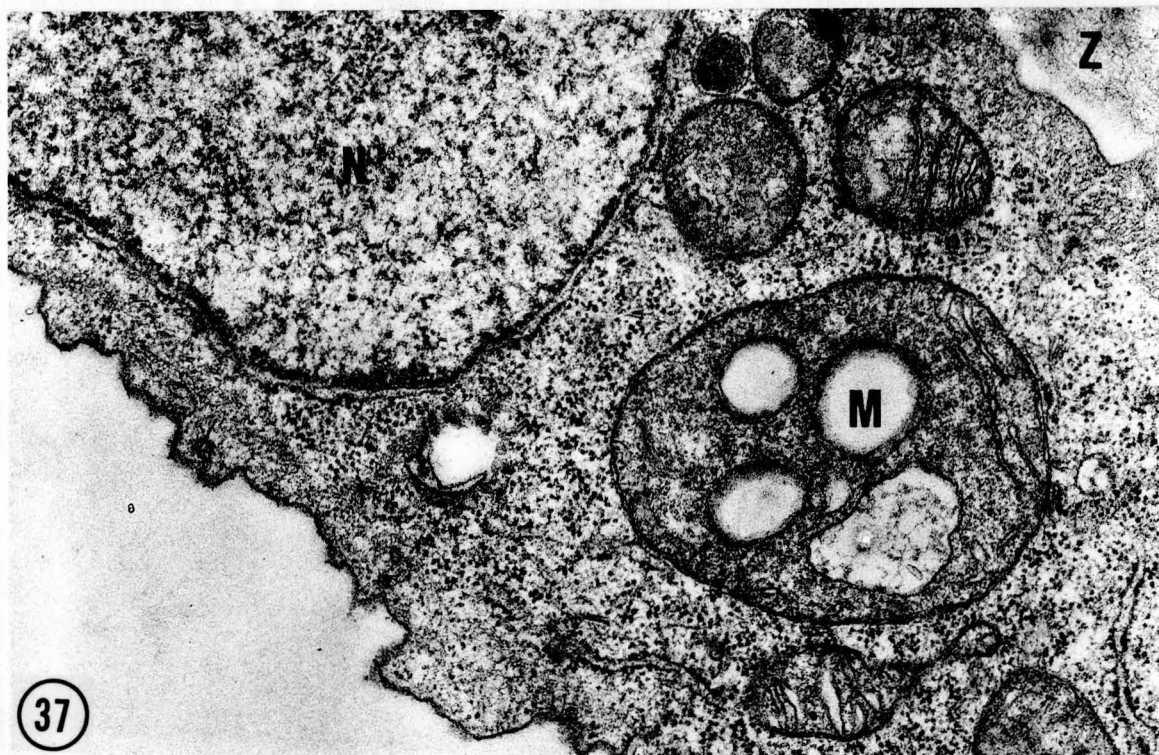


PLATE XXIV

EXPLANATION OF FIGURE

Figure 39. Trophoblast cell of a blastocyst grown 92 hours in vitro from the late two-cell stage. The plane of section has passed tangentially to the nucleus (N). Several mitochondrial shapes (M) are present. Microvilli are elongate on the basal or blastocelic surface (B). This particular trophoblast cell contains a relatively large number of ribosomes. Arrow, micropinocytotic caveola; Z, zona pellucida; f, microfilaments. X30,000.

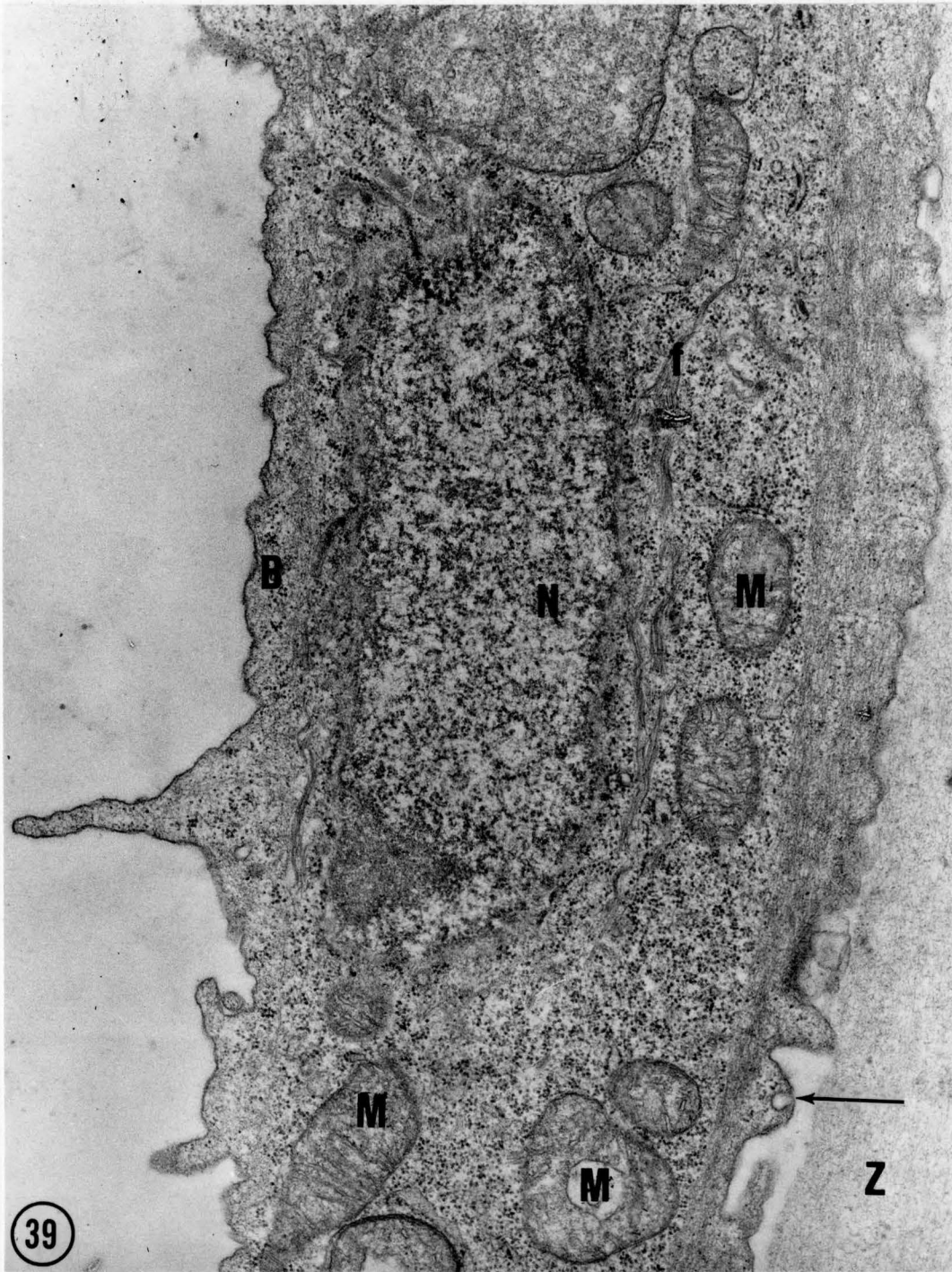


PLATE XXV

EXPLANATION OF FIGURE

Figure 40. This is an electron micrograph of one of the more normal-appearing inner mass cells found in embryos cultured 92 hours in vitro from the late two-cell stage. There appear to be more ribosomes than are found in cells of embryos cultured 72 hours in vitro, and most of the fibrous plaques have disappeared. However, few ribosomes line the outer nuclear membrane. Y, myelin-like figure; arrow, nuclear pore; N, nucleus; E, endoplasmic reticulum; A, autophagic vacuole. X30,000.

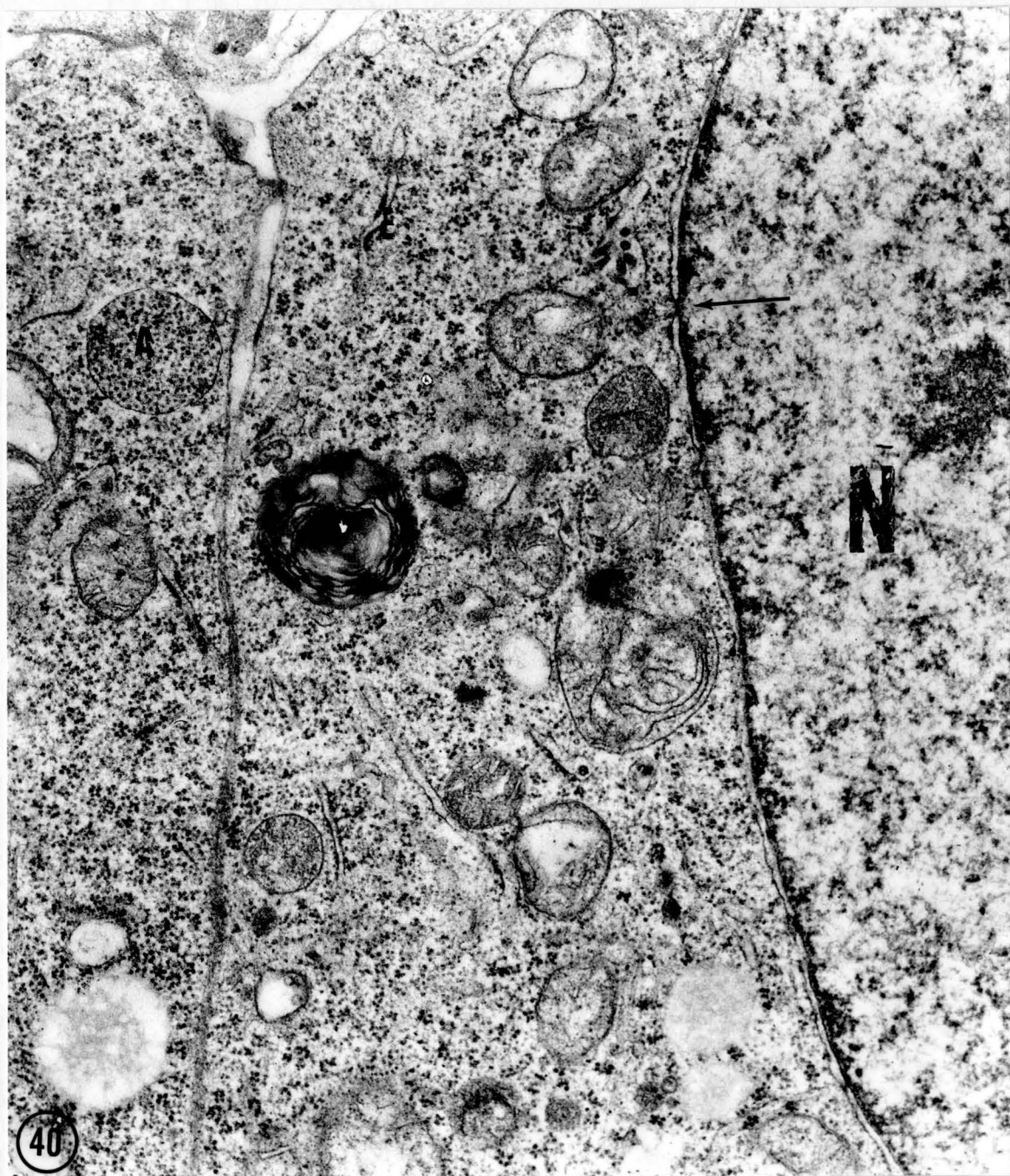


PLATE XXVI

EXPLANATION OF FIGURE

Figure 41. Inner mass cell of an embryo cultured 92 hours in vitro from the late two-cell stage. The large number of autophagic vacuoles (A) indicate that the cell is undergoing autolysis. Nevertheless, the nuclear and cell membranes are still intact (arrows). A lysosome-like structure similar to those observed in cells of blastocysts grown 72 hours in vitro is also observed in the cytoplasm of this inner mass cell (compare with Figure 34). X30,000.

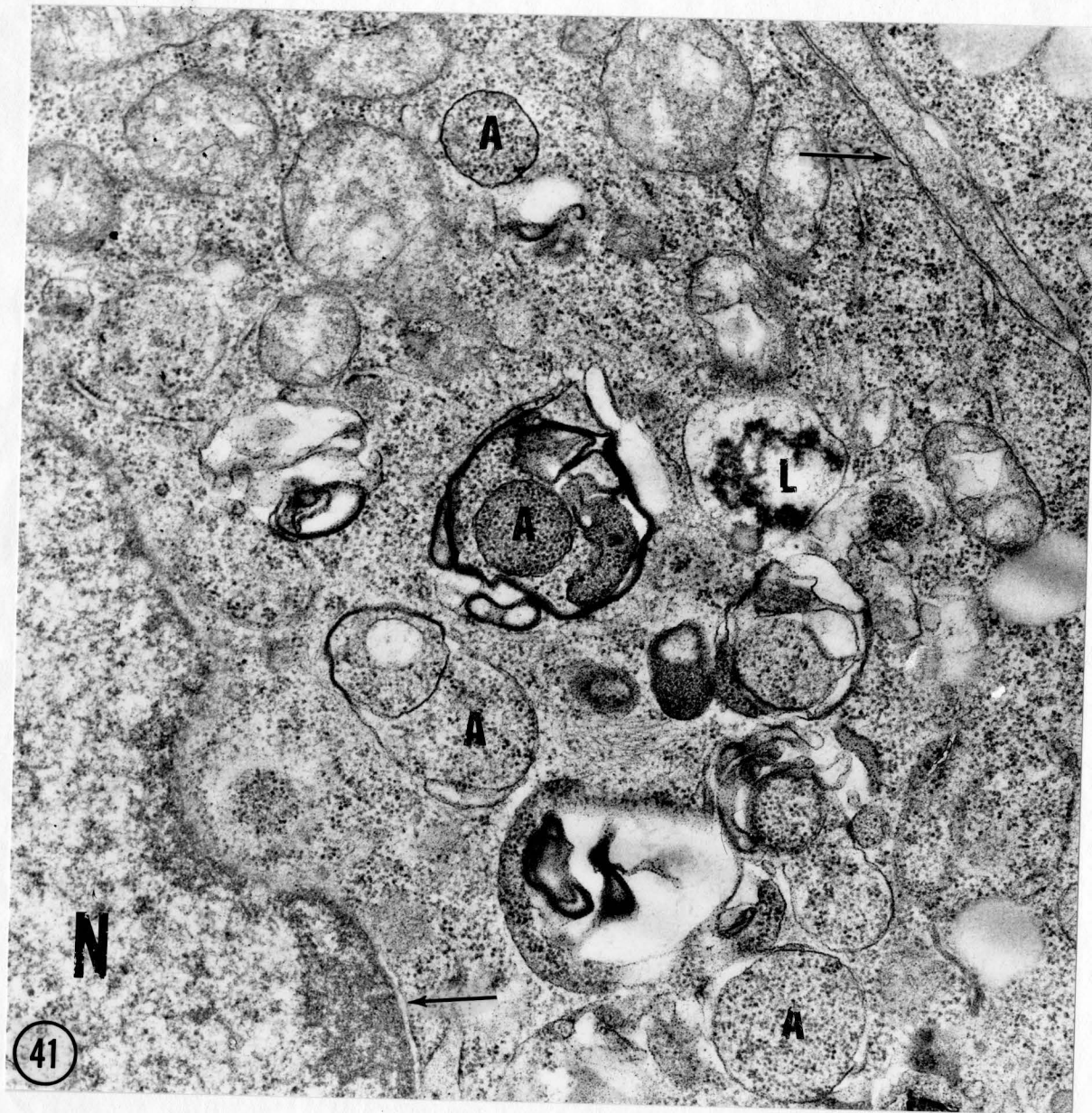


PLATE XXVII

EXPLANATION OF FIGURES

Figure 42. An elongate, rather well-differentiated nucleolus found within an inner mass cell of a blastocyst grown 92 hours in vitro. This nucleolus is in contact with the inner nuclear membrane. Note the fibrillar (F) and granular (G) areas of the nucleolus. X30,000.

Figure 43. Two inner mass cells of a blastocyst grown 92 hours in vitro from the late two-cell stage. Note the swollen mitochondria (m) which contain several intracristal vacuoles. A moderate number of ribosomes are present in the cytoplasm, as well as crystalloids (C) and autophagic vacuoles (L). Note the eccentric position of the nucleus (N) of the cell at the right of the figure. X30,000.

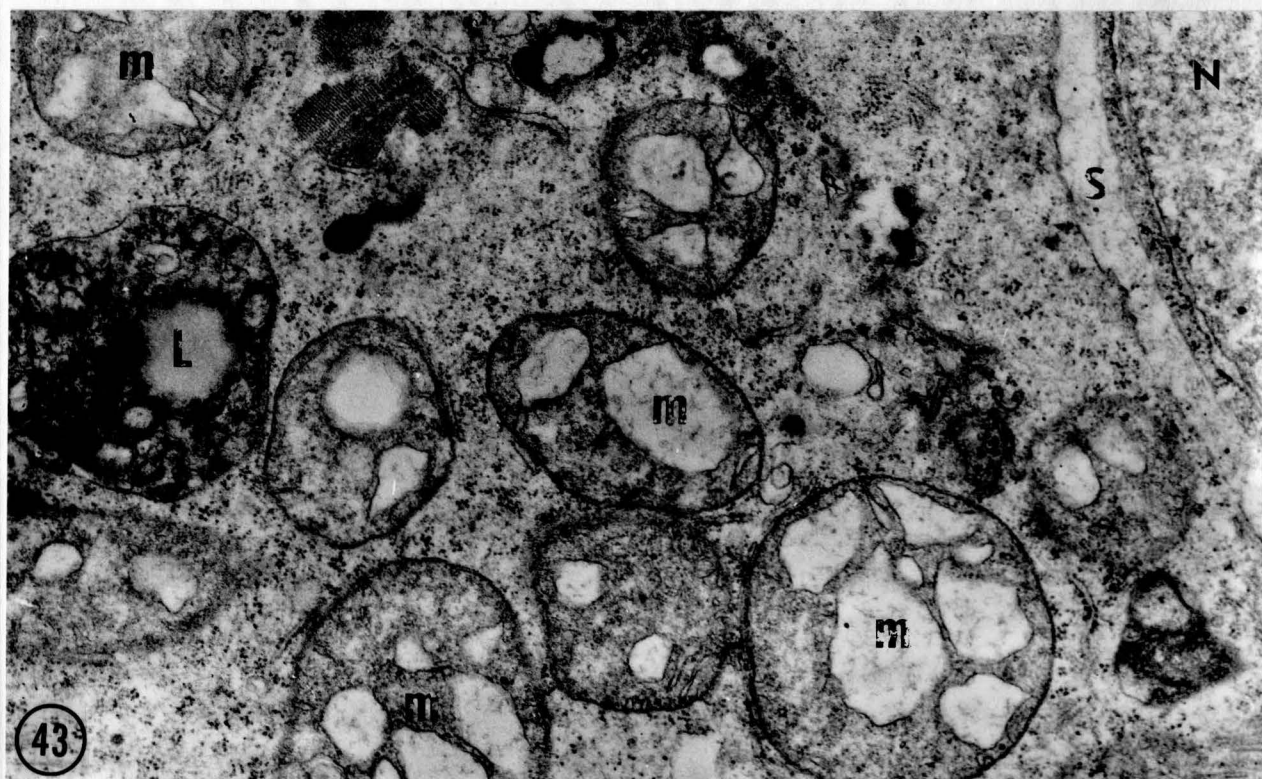
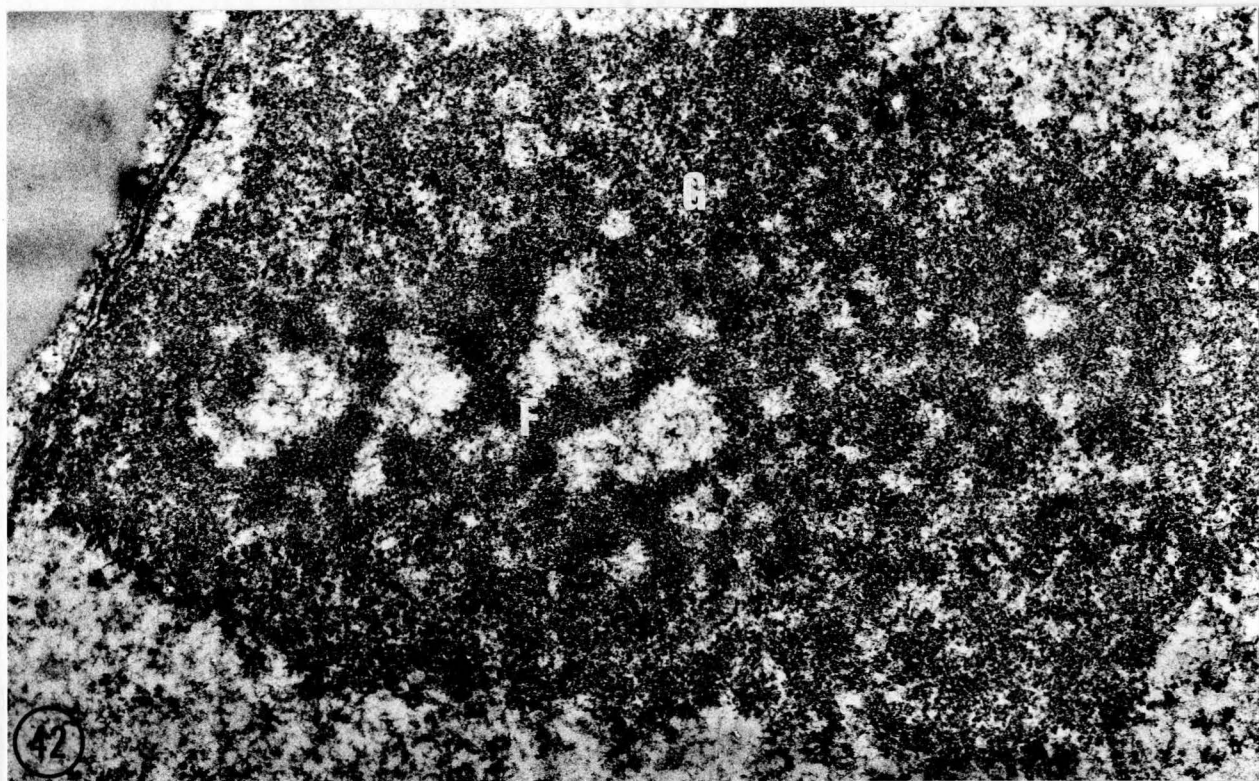


PLATE XXVIII

EXPLANATION OF FIGURE

Figure 44. Normal-appearing inner mass cells of a blastocyst developed 92 hours in vitro from the late two-cell stage. There appear to be fewer ribosomes in these cells than in inner mass cells of the in vivo developed late blastocyst. Note the large number of crystalloids (C) and fibrous strands (F) which are rarely observed in the in vivo developed blastocyst. N, nucleus; M, mitochondrion. X30,000.

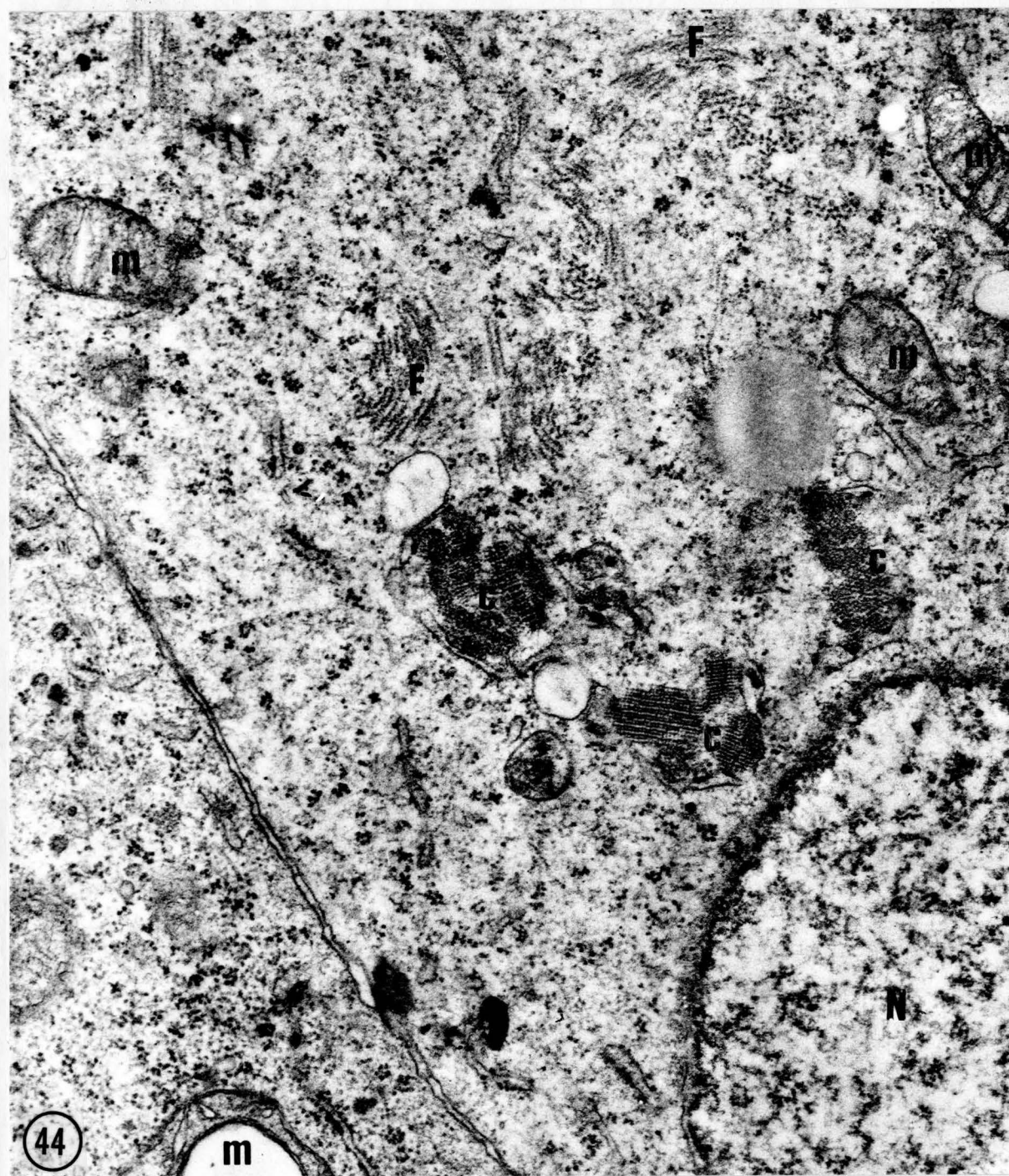


PLATE XXIX

EXPLANATION OF FIGURE

Figure 45. Inner mass cell (of a blastocyst grown 92 hours in vitro) in an advanced stage of autolysis. Autophagic vacuoles (A) and swollen mitochondria (m) fill the cytoplasm. The numerous fibrous strands (f) still present in the cytoplasm indicate that autolysis commenced somewhat earlier. The nucleus is eccentric, and one of the two nucleoli is completely rounded or clumped against the inner nuclear membrane (X). Little heterochromatin appears along the inner nuclear membrane or in foci in the nucleoplasm. S, intercellular space. X22,000.

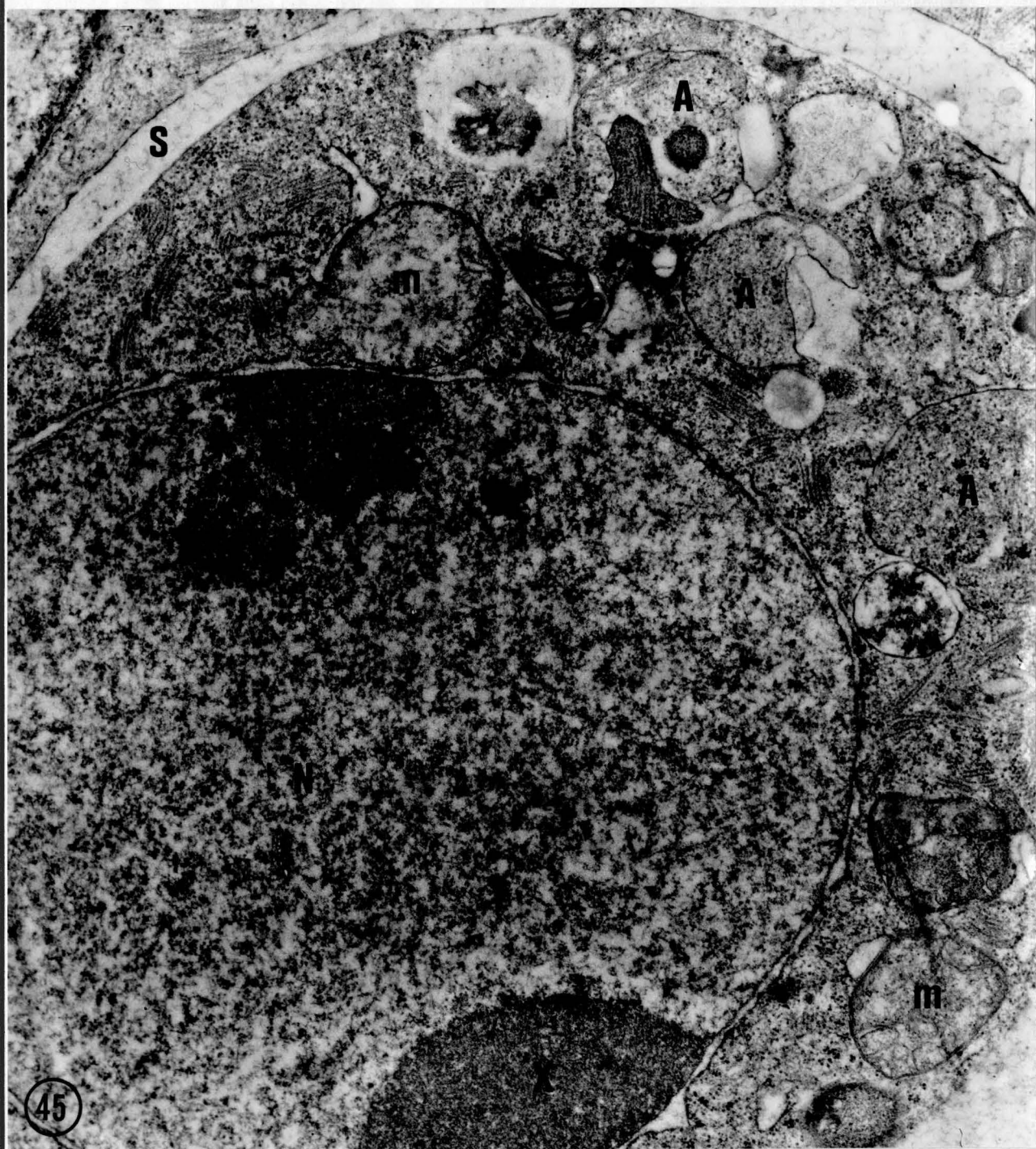


PLATE XXX

EXPLANATION OF FIGURES

Figure 46. An autolytic inner mass cell of a blastocyst grown 92 hours in vitro from the late two-cell stage. The nucleus is eccentric and the nucleolus (N) appears clumped against the inner nuclear membrane. The cell membrane appears relatively smooth, but the membrane of the cell on the right displays discontinuities. Note the large intercellular space (S) formed as the result of cytoplasmic contraction. X22,000.

Figure 47. Inner mass cell of an embryo cultured 92 hours in vitro. This cell appears to be in an advanced stage of autolysis. Although the cytolemma appears intact, the nuclear membrane has disappeared. The large, round, electron-dense structure (N) most likely represents a clumped nucleolus. Strands of rough endoplasmic reticulum are aligned parallel to one another and rounded spheres (arrows), similar to microsomes, appear to be formed from elements of the rough endoplasmic reticulum (arrows). X30,000.

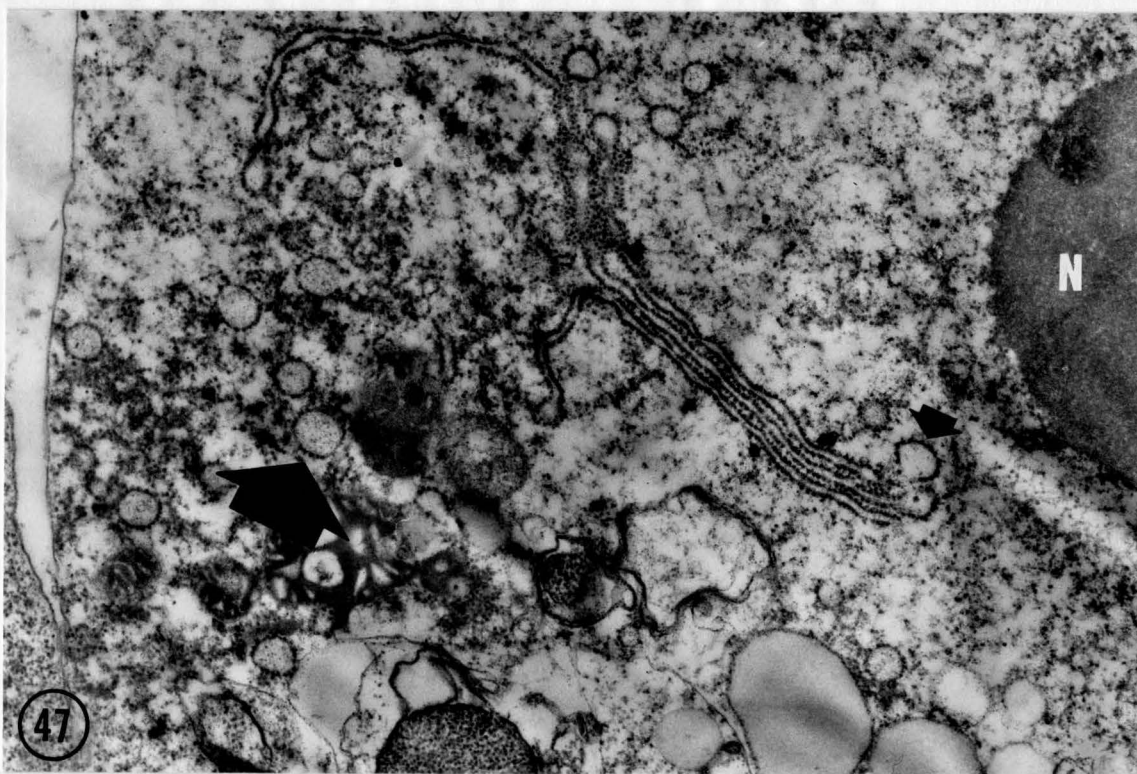


PLATE XXXI

EXPLANATION OF FIGURE

Figure 48. Embryo cultured 26 hours in vitro from the late morula-early blastocyst stage. The cell in the upper portion of the electron micrograph is in mitosis. Note the clustered elements of endoplasmic reticulum at one pole of the cell (ER). Ribosomes are ubiquitous, as in cells of the in vivo developed late preimplantation blastocyst. Fibrous plaques have disappeared. Mitochondria (M) display an electron-dense matrix and many contain intra-cristal vacuoles. Note the intimate association of the rough endoplasmic reticulum with the mitochondria. This type of association is typically observed in cells of all blastocysts cultured in vitro. G, Golgi complex; C, chromosomes. X12,000.

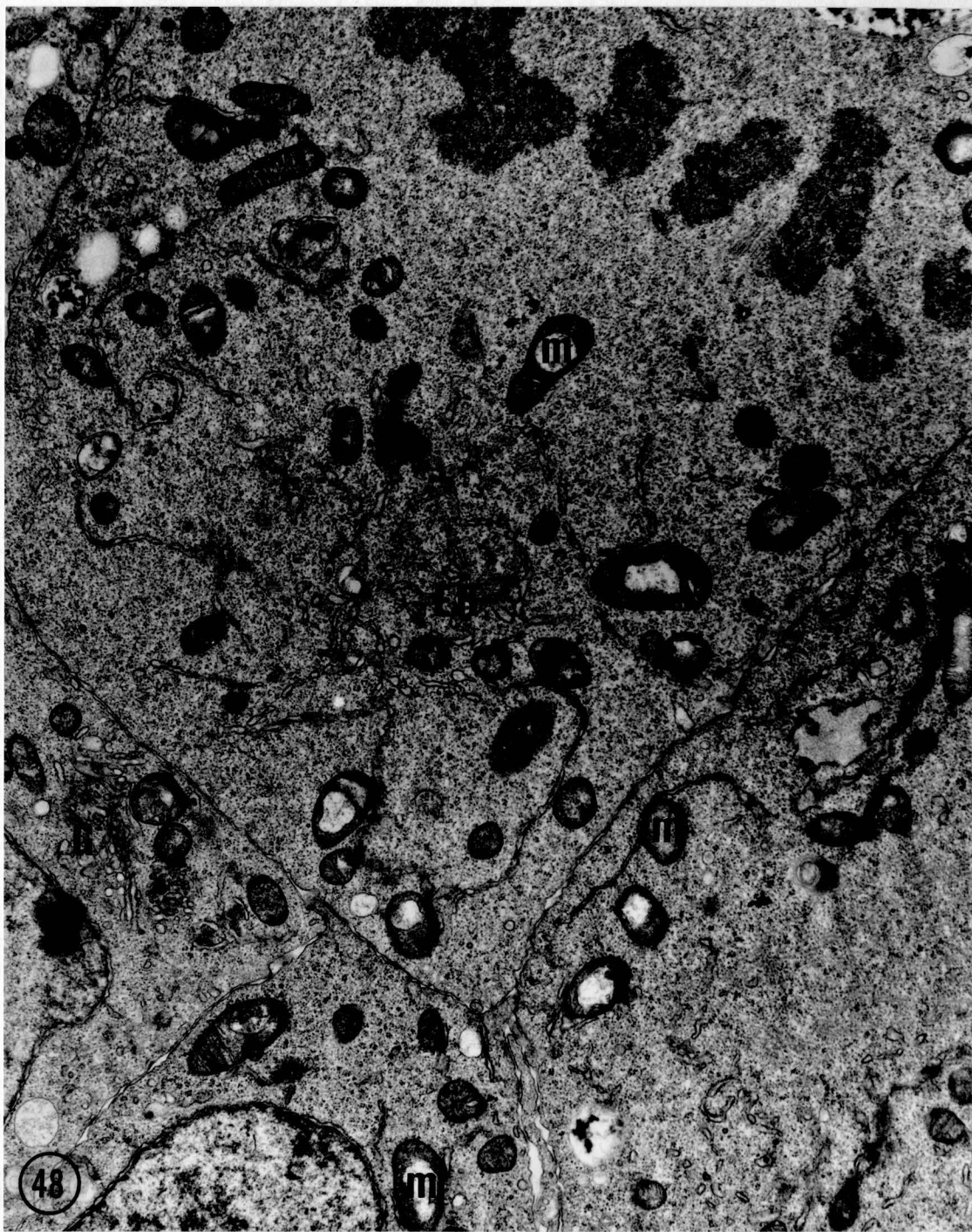


PLATE XXXII

EXPLANATION OF FIGURES

- Figure 49. A section through a trophoblast cell (T) and endoderm cell (E) of a mouse blastocyst cultured 26 hours in vitro from the late morula stage. A sperm tail remnant can be observed in the trophoblast cell, C, crystalloid. X13,000.
- Figure 50. A slightly oblique section through the neck region of the same sperm tail observed in Figure 49. Arrow points to the limiting membrane which encircles the sperm tail remnant. V, vesicles; N, nucleus. X30,000.

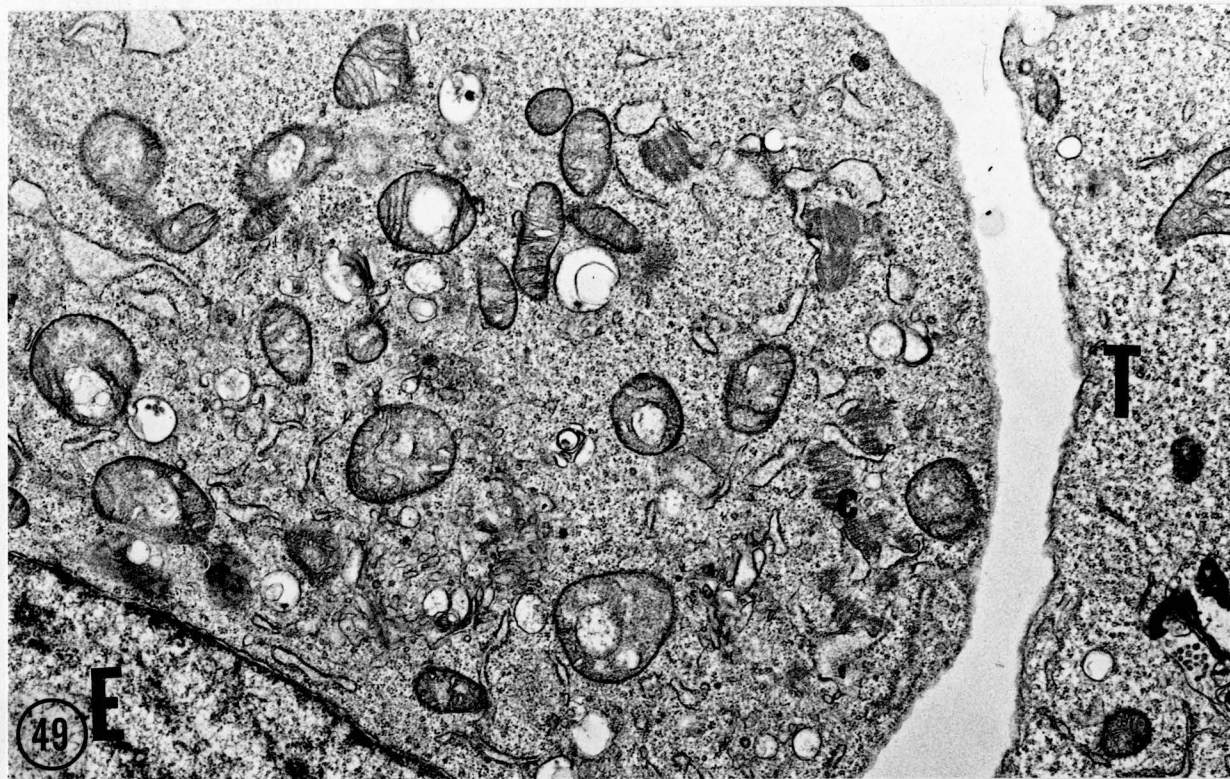


PLATE XXXIII

EXPLANATION OF FIGURES

Figure 51. An oblique section through the neck of a sperm tail remnant in a trophoblast cell of an embryo cultured 26 hours *in vitro* from the late morula-early blastocyst stage. The sperm tail is surrounded by a limiting membrane. Notice the Golgi apparatus (G). N, nucleus; M, mitochondrion; R, ribosomes. X54,000.

Figure 52. Trophoblast cell of the same blastocyst observed in Figure 51. This is an electron micrograph of a nearly cross-section through the middle piece of a sperm tail remnant. Notice the limiting membrane which surrounds the inclusion. The arrow points to a sperm mitochondrion, which is smaller than the adjacent mitochondria of the embryo proper. Note the electron-dense, lysosome-like structure (L) which has coalesced with the membrane which surrounds the sperm tail. G, Golgi complex; N, nucleus. X30,000

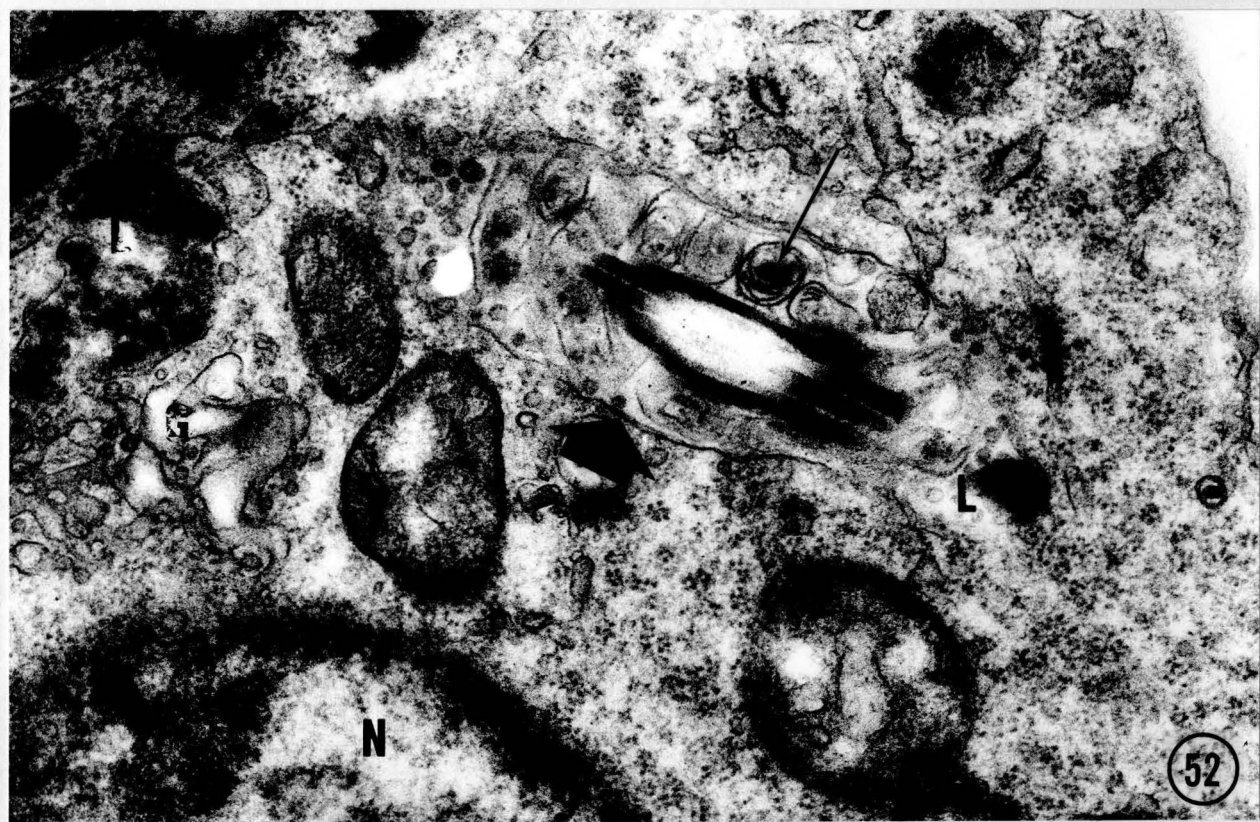
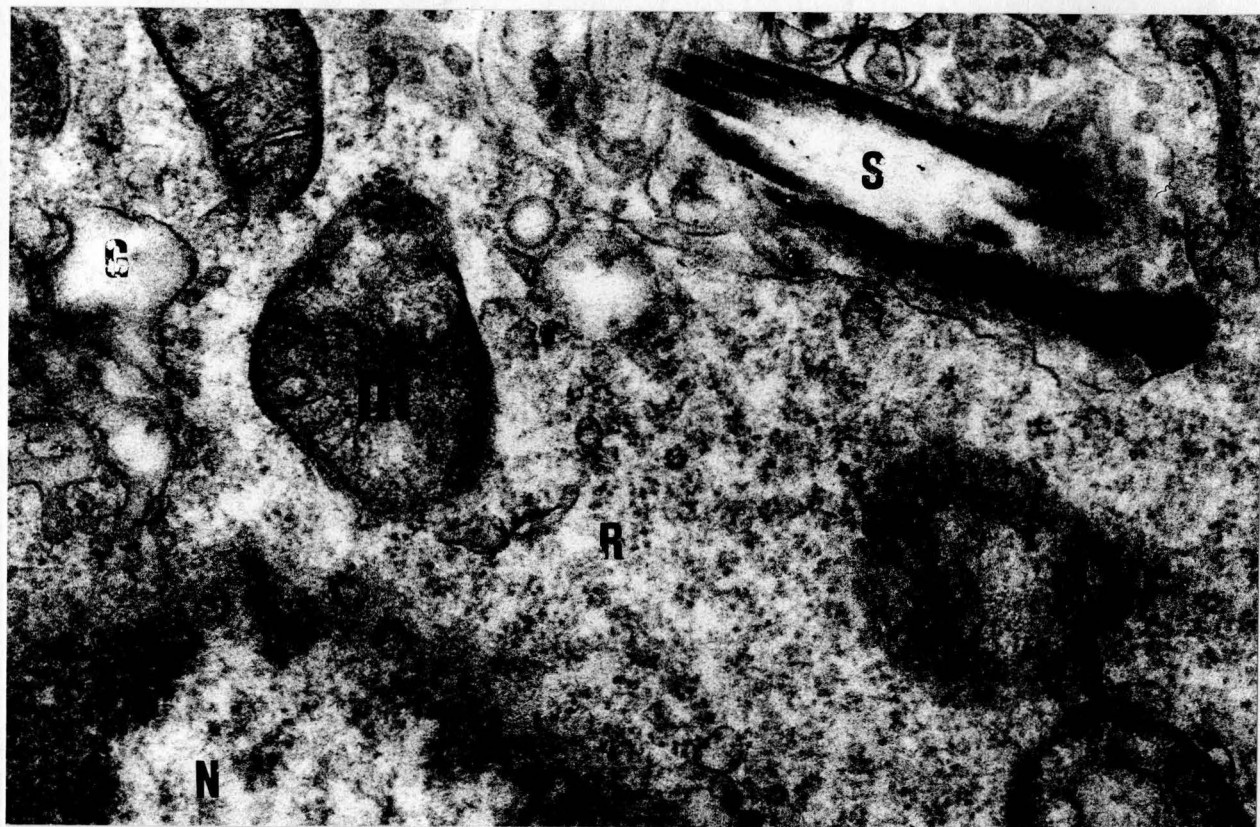


PLATE XXXIV

EXPLANATION OF FIGURE

Figure 53. A trophoblast cell and two inner mass cells of an embryo cultured 26 hours in vitro from the late morula stage of preimplantation development. The cells are heavily laden with ribonucleoprotein particles. Condensed chromatin or heterochromatin (H) is found lining the inner nuclear membrane, as well as in foci in the nucleoplasm. The trophoblast cell (on the right) contains a well-differentiated nucleolus, and its Golgi complex is moderately distended (G). Note the electron-dense lysosome-like bodies (L) similar to those found in cells of the in vivo developed blastocyst. V, cytoplasmic vesicles; C, crystalloids. X14,000.



PLATE XXXV

EXPLANATION OF FIGURES

Figure 54. Crystalloid inclusions in an inner mass cell of a blastocyst grown 26 hours in vitro from the late morula stage. Note the close association with the elements of the rough endoplasmic reticulum. G, Golgi complex. X30,000.

Figure 55. Two inner mass cells of a blastocyst grown 26 hours in vitro from the late morula-early blastocyst stage. The Golgi complex (G) is moderately distended. X22,000.

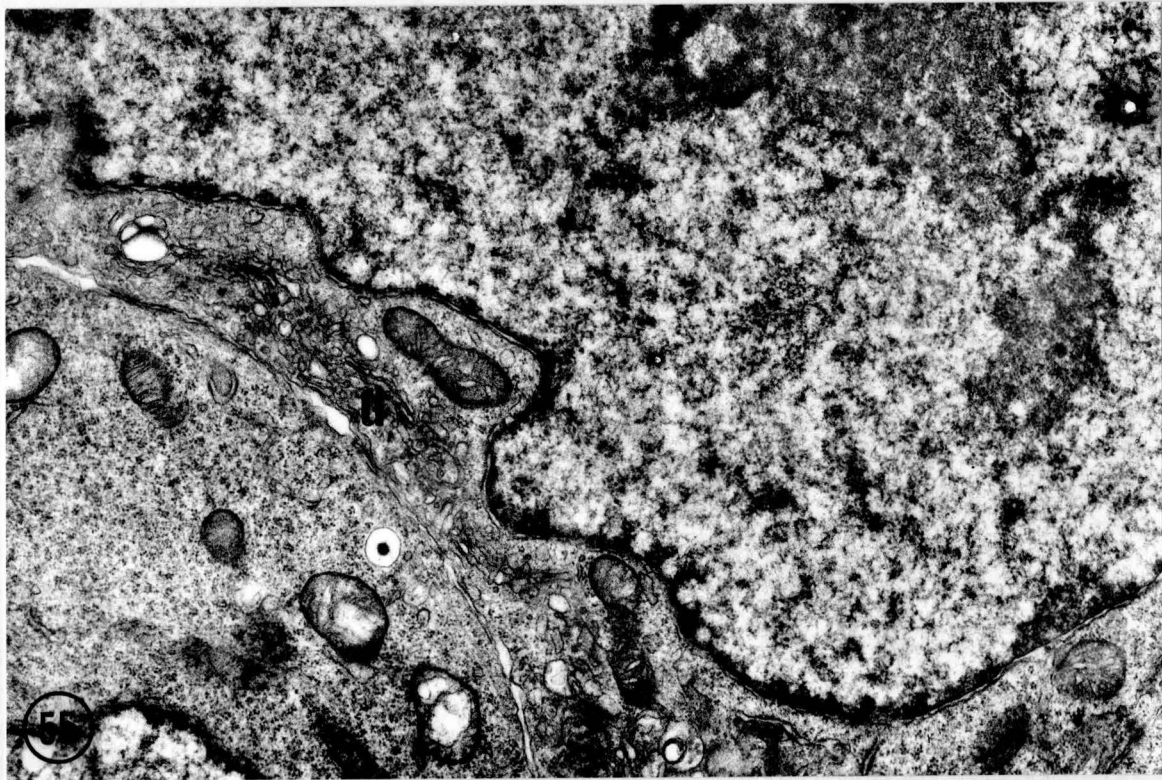


PLATE XXXVI

EXPLANATION OF FIGURE

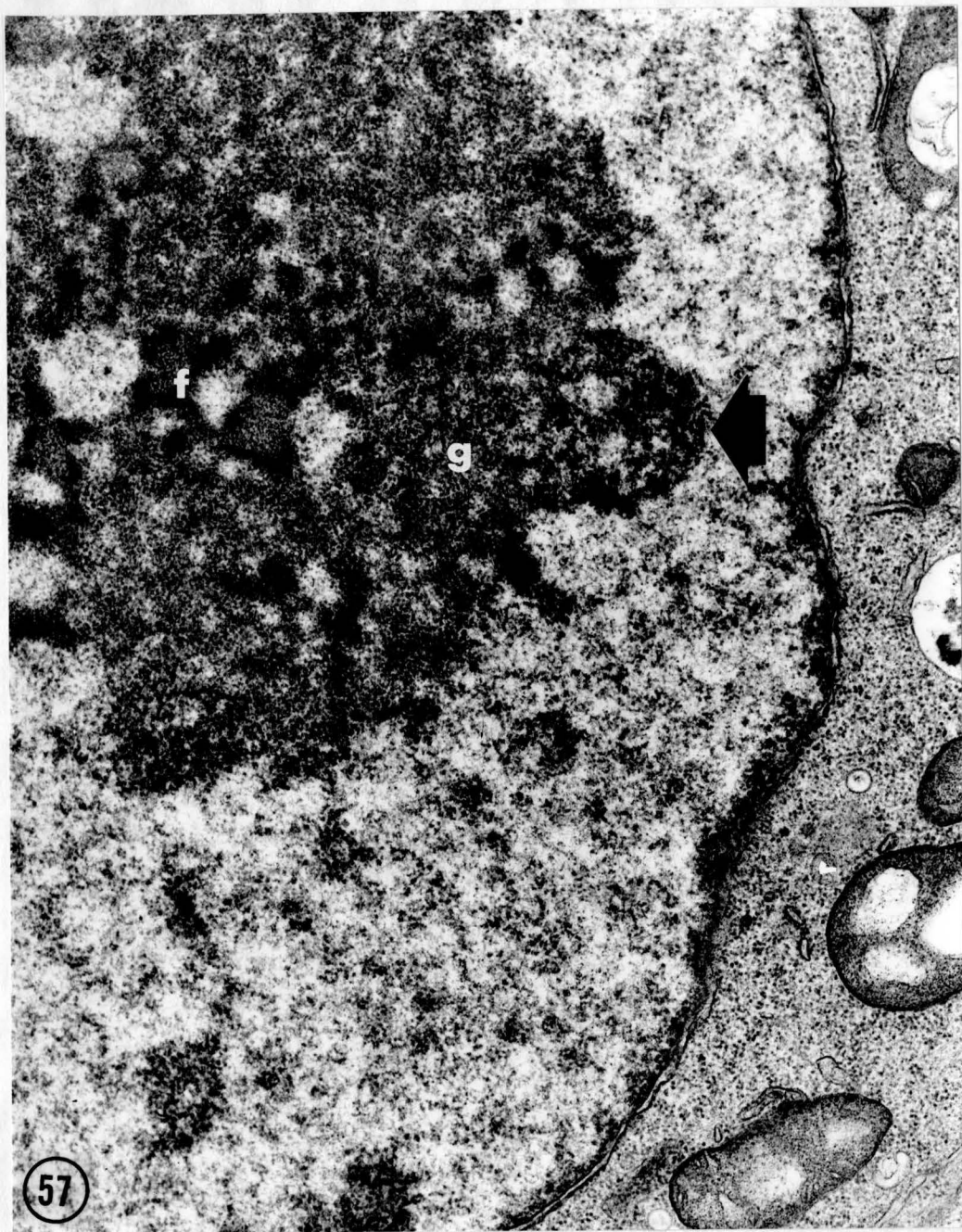
Figure 56. Trophoblast and inner mass cells of an embryo grown 26 hours in vitro from the late morula stage. Moderate amounts of heterochromatin, similar to that observed in cells of in vivo developed late preimplantation mouse blastocysts, lines the inner nuclear membranes and is found in foci throughout the nucleoplasm. One inner mass cell contains a well-differentiated nucleolus (No). Mitochondria have an electron-dense matrix, and several contain intracrystal vacuoles. As in inner mass and trophoblast cells of the in vivo late preimplantation mouse blastocyst, these cells are heavily laden with ribosomes (Compare with Figure 6). G, Golgi complex. X14,000.



PLATE XXXVII

EXPLANATION OF FIGURE

Figure 57. Inner mass cell of blastocyst developed 26 hours in vitro from the late morula stage. The nucleolus is large, compact, and well-differentiated, and it contains fibrillar (f) and granular (g) areas. Condensed chromatin or heterochromatin is associated with the nucleolus (arrow) and it also lines the inner nuclear membrane. X30,000.



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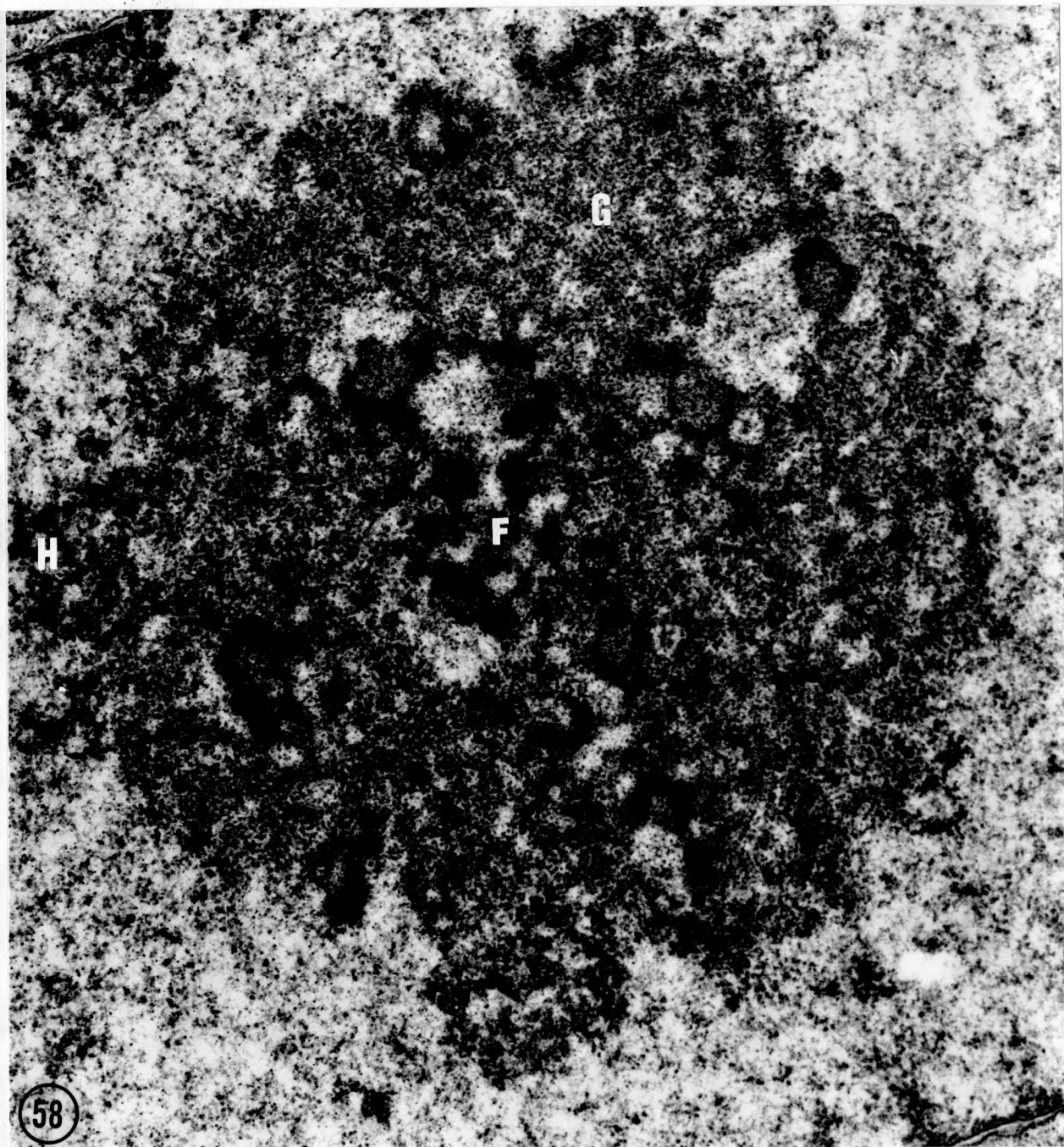


PLATE XXXVIII

EXPLANATION OF FIGURE

Figure 58. Nucleus of inner mass cell of an embryo cultured 26 hours in vitro from the late morula-early blastocyst stage of preimplantation development. The well-differentiated nucleolus found in this cell consists of fibrillar (F) and granular (G) areas with condensed chromatin associated at its periphery. H, heterochromatin. X 54,000.

PLATE XXXIX

EXPLANATION OF FIGURE

Figure 59. Trophoblast cell of a blastocyst grown 26 hours in vitro from the late morula-early blastocyst stage of preimplantation development. Notice the close relationship between the mitochondria and the channels of rough endoplasmic reticulum (arrows). Mitochondria (M) have an electron-dense matrix and some contain intracrystal vacuoles. Arrow, micropinocytotic caveola. X 48,000.



PLATE XL

EXPLANATION OF FIGURE

Figure 60. An endoderm cell undergoing mitosis in a blastocyst grown 26 hours in vitro from the late morula-early blastocyst stage. One can observe a widely dilated cistern of rough endoplasmic reticulum (ER), as well as several smaller tubules which are intimately associated with mitochondria (arrow). Ribosomes are abundant (R), and no fibrous strands are present. C, chromosomes. X14,000.

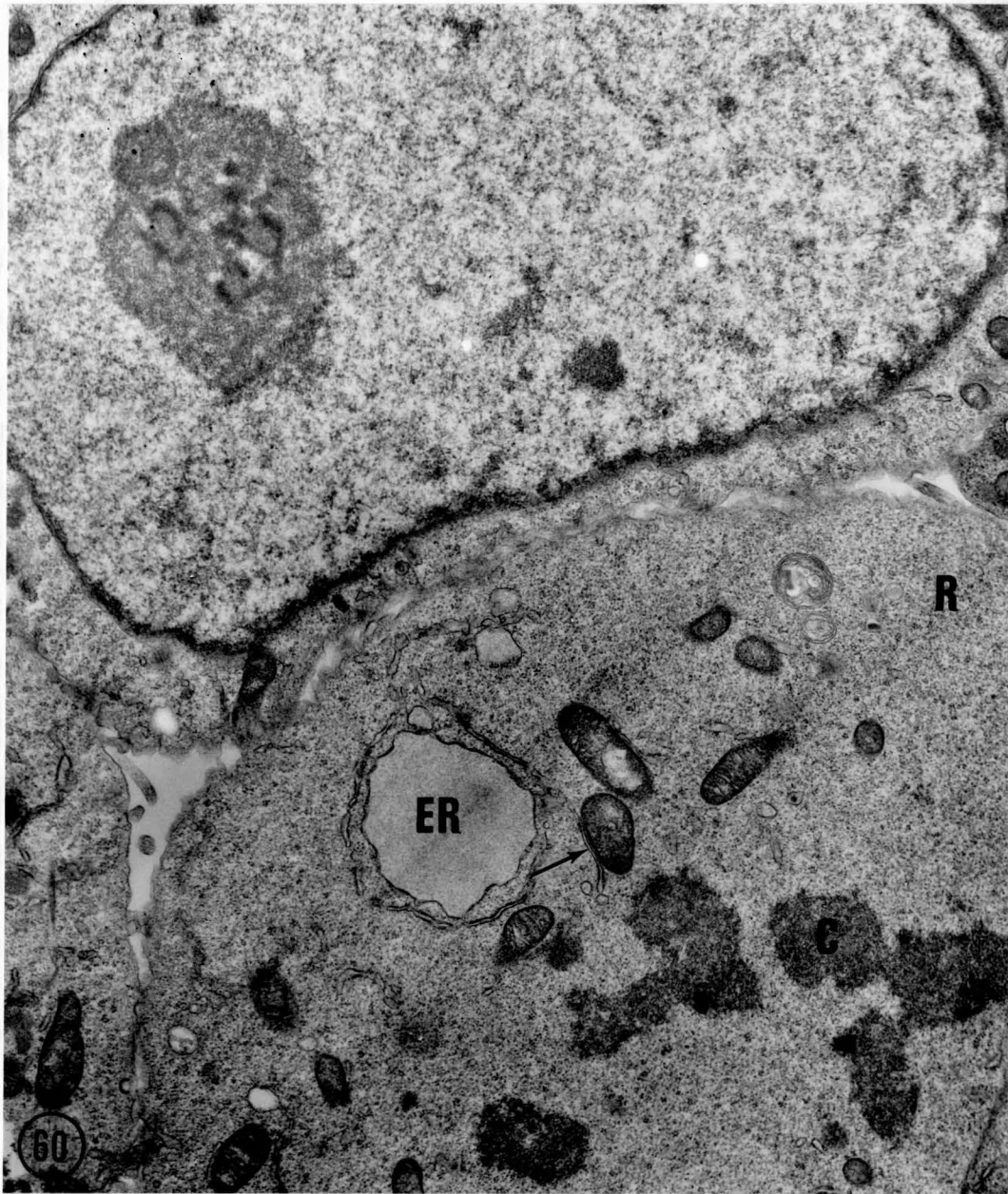
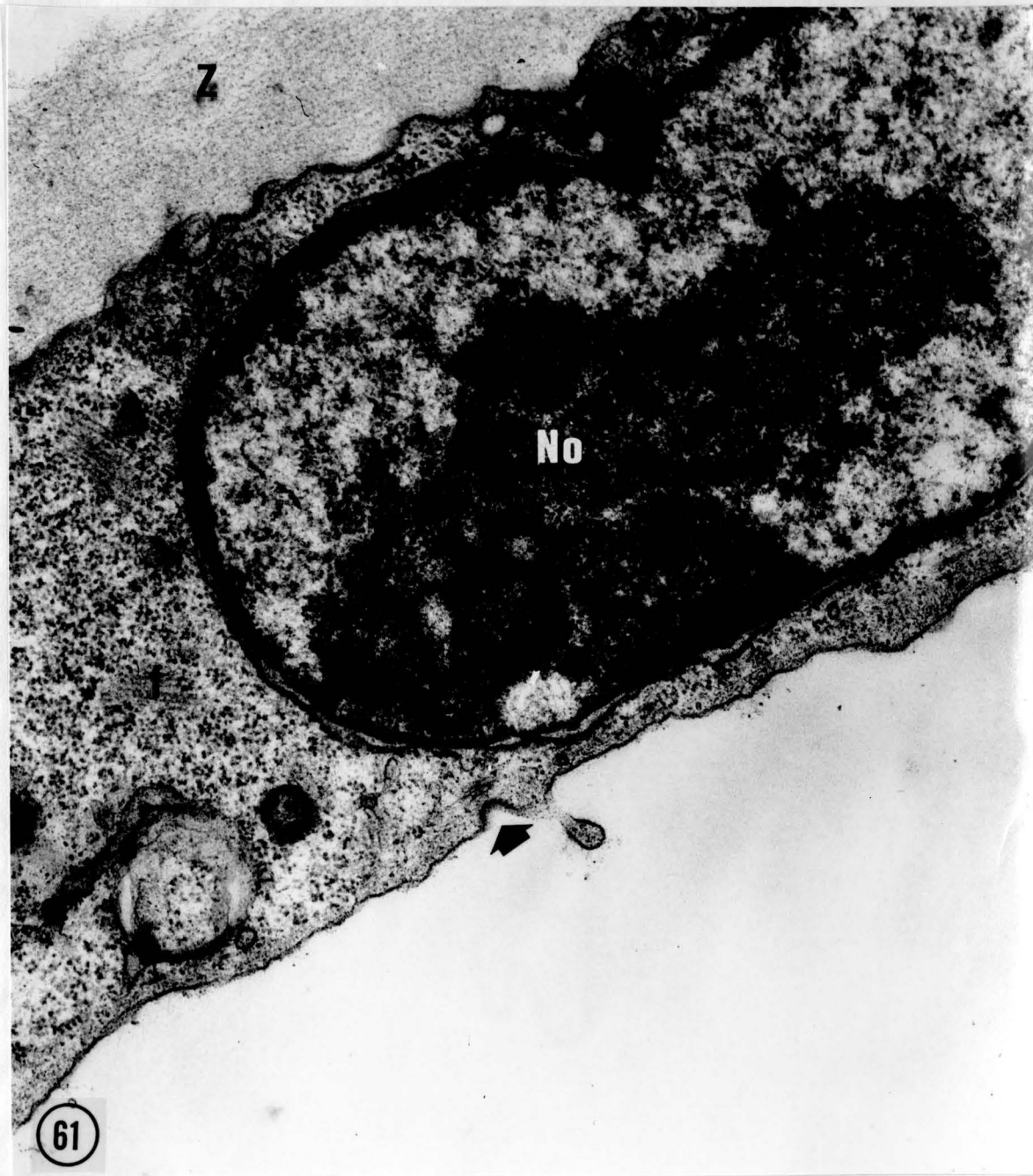


PLATE XLI

EXPLANATION OF FIGURE

Figure 61. Trophoblast cell of a blastocyst grown 26 hours *in vitro* from the late morula-early blastocyst stage of preimplantation development. The nucleus contains an elongate, well-differentiated nucleolus (No), as well as condensed chromatin. Note the distended perinuclear cisterna which is similar to the perinuclear cisternae which are present in inner mass and trophoblast cells of the *in vivo* developed late preimplantation mouse blastocyst. Moreover, the outer nuclear membrane is studded with ribosomes. A few fibrous strands are observed in the cytoplasm (f). Z, zona pellucida; arrow, micropinocytotic caveola. X30,000.



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APPROVAL SHEET

The dissertation submitted by Hal D. McReynolds has been read and approved by members of the faculty of the Graduate School of Loyola University of Chicago.

The final copies have been examined by the chairman of the thesis committee and the signature which appears below verifies the fact that any necessary changes have been incorporated, and that the dissertation is now given final approval with reference to content, form and accuracy.

The dissertation is therefore accepted in partial fulfillment of the requirements for the Degree of Doctor of Philosophy.

06/22/70

Date

Robert Healy

Signature of Advisor